

Novel Cryopreservation Strategies for Cell-Therapies and Pre-Clinical Research

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by Rita Malpique

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Front Cover

By Rita Malpique. Composite image of the main cell models used for the development of novel strategies for complex cell systems. Clockwise: Scanning electron microscopy of enterocyte-like differentiated Caco-2 monolayers; Immunofluorescence microscopy of neuronal differentiated N2a cells with labeled low molecular weight neurofilament protein; Immunofluorescence microscopy of alginate encapsulated brain rat neurospheres with labeled glial fibrillary acidic protein and β -tubulin-III protein; Phase-contrast microscopy of a hESC colony.

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Foreword

The present thesis dissertation is the result of four years of research at the Animal Cell Technology Unit of ITQB-UNL/IBET, Oeiras, Portugal, under the supervision of Dr. Paula M. Alves and at the Biophysics and Cryotechnology Department, Fraunhofer IBMT, St. Ingbert, Germany, under the co-supervision of Prof. Dr. Heiko Zimmermann. It gave me the opportunity to be introduced to the challenging field of cryobiology and allowed me to explore the culture and cryopreservation of complex cell systems, such as cell lines with the capacity of differentiation, cell monolayers, primary brain cell cultures and human embryonic stem cells.

This thesis intends to explore novel cryopreservation strategies by addressing specific steps within the cryopreservation workflow, in order to understand and improve the current inefficient cryopreservation methodologies for complex cell systems, namely human embryonic stem cells.

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Abstract

The expanding need for complex biologics for therapeutic applications, *in-vitro* pharmacology and toxicology studies and fundamental research demands the production of banks of well-characterized and safety-tested stocks of a large number of cell/tissue samples. This implies the development of effective cryopreservation methodologies that can cope with process scalability and automation and must reflect the biological and physical properties of the cells as these can be significantly altered by the process. Currently, efficient cryopreservation protocols are established for a wide variety of cell types, which are frozen as single-cell suspensions yielding high survival rates. However, such techniques fail to provide acceptable recovery rates for complex systems such as two-dimensional (2-D) monolayer cell cultures, three-dimensional (3-D) aggregates, tissues and organs, or more sensitive systems such as primary cultures or human embryonic stem cells (hESC).

The main focus of this thesis is the development and validation of effective, consistent, current Good Manufacture Practices (cGMP)-compliant, scalable and automatable cryopreservation protocols for complex cell systems, which can generate sufficient quantities of well-characterized samples for research, therapeutic and/or pharmacological applications. Specifically, cryopreservation strategies for functional 2-D monolayers and primary cultures of 3-D aggregates were developed, being the final aim to obtain improved cryopreservation protocols for hESC. The rationale behind the selection of 2-D and 3-D cell systems was based on the important characteristics they share with the hESC colonies, namely the post-thaw maintenance of cell specific function and cell-cell/cell-matrix interactions as major requirements for a successful cryopreservation. To achieve this aim, an integrated approach was developed by addressing specific

steps within the general cryopreservation workflow, namely the pre-freezing conditions, freezing method and post-thaw recovery methodologies.

In **Chapter I**, a general introduction to the theme of cryopreservation is presented, with particular relevance given to the impact of the different parameters within the cryopreservation workflow on post-thaw cell recovery. A special focus is given to the state-of-the-art regarding the current drawbacks of this technology when applied to different cell systems, namely 2-D and 3-D cell systems and hESC, and proposed strategies to overcome them.

In **Chapter II**, a novel strategy for cell cryopreservation, based on miniaturisation and automation, was validated, which is of major importance on the establishment of large cell banks. This was done by studying the effect of pre-freezing conditions on the post-thaw viability of Caco-2 colon adenocarcinoma cells, which differentiate spontaneously after long-time in culture, when cryopreserved through slow-rate freezing as single-cell suspensions. Namely, reduction of the cryovessel volume, from the traditionally used 1 ml cryovials to the 25 μ l well of the cryo-microsubstrates tested, and choice and method of addition of the cryoprotectant (CPA) were tested. Moreover, in order to determine the important parameters which must be investigated following cryopreservation, different properties were evaluated, such as cells capacity to proliferate and differentiate post-thawing. The results have shown that the reduction of the cryodevice did not affect the post-thawing cell membrane integrity neither cell function nor differentiation potential, which is a fundamental requirement for further cell-therapeutic utilizations.

In **Chapters III and IV**, cryopreservation of 2-D and 3-D cell systems with a wide range of applications in pre-clinical research and high-throughput screening was addressed taking into account the specific challenges concerning each system.

Ultra-high viscous (UHV), clinical-grade alginate was used for cell entrapment prior to cryopreservation as the main strategy for avoiding monolayer detachment, colony disintegration and lost of cell-cell and cell-matrix contact and due to its potential to be used as a xeno-free matrix for cell therapy. In order to avoid the observed post-thaw delayed cell death and aiming at a cGMP-approved compliant cryopreservation protocol, the protein- and serum-free commercial solution supplemented with apoptosis inhibitors, CryoStor™, was compared with standard cell culture media as the vehicle solution for the CPA. In **Chapter III**, cryopreservation through slow-rate freezing of cells attached to well-plate's surfaces was investigated. Two cell lines, neuroblastoma N2a and colon adenocarcinoma Caco-2, were used due to their distinct structural and functional characteristics. It was shown that the combination of gel entrapment and CryoStor™ solution results in cell specific responses to cryopreservation that can lead to optimized protocols as shown for N2a cells. Although entrapment beneath an alginate layer was found to improve cell recovery by minimizing membrane damage and cell detachment after thawing, the use of CryoStor™ solution was crucial for full recovery of metabolic activity and differentiation capacity within 24 hours post-thawing; in this case, entrapment beneath an UHV alginate layer did not confer further protection to cryopreserved Caco-2 cells but was crucial for maintenance of attachment and integrity of N2a neuronal networks.

Chapter IV describes the development of an integrated strategy allowing for long-term culture and cryopreservation of primary cultures of brain neurospheres with high viability, reduced recovery time post-thawing and significantly less fragmentation. The encapsulation process did not affect cell viability central metabolism, neither cell differentiation nor the cell extensions into cell networks usually observed between neurons-astrocytes within the neurospheres structure. Furthermore, the use of serum-free CryoStor™ solution provided further protection for both non-encapsulated and encapsulated aggregates when

compared to serum-supplemented culture medium as the cryopreservation medium. Results with both cell models have shown that entrapment within UHV alginate and the use of CryoStorTM solution are successful approaches to improve the post-thaw survival of the 2-D and 3-D cell-models after cryopreservation through slow-rate freezing, which enabled the retention of cell membrane and whole monolayer/aggregate integrity, metabolic activity and cell specific function/differentiated state, a major requirement for pre-clinical research and therapeutic applications.

In **Chapter V**, the cryopreservation steps studied and optimized in Chapters III and IV were combined with the aim of developing an integrated cryopreservation strategy for hESC. When applied to hESC, the improved slow-rate freezing protocols developed for the 2-D and 3-D cell models have proven not to be suitable for the successful cryopreservation of the colonies. Thus, different approaches were evaluated, namely the use of vitrification instead of slow-rate freezing as the freezing method. Vitrification of adherent colonies yielded significantly higher hESC recovery rates when compared with the slow-rate freezing approach: high survival and low colony fragmentation were found immediately post-thawing and during further culture until cell passage. Furthermore, such protocol resulted in low differentiation rates and maintenance of pluripotency post-thawing. This novel surface-based vitrification method may facilitate the development of a high-throughput cryopreservation process for hESC and reduce the time required to amplify frozen stocks, thus supporting the widespread use of these cells in research, clinical or pharmacological applications.

Chapter VI consists of a general discussion, where future perspectives and main conclusions of the work are presented.

This thesis contributes substantially to the establishment of effective cryopreservation methodologies for complex cell systems, namely 2-D monolayers, 3-D aggregates and hESC colonies, with the identification and analysis of the main bottlenecks in process development and proposed effective strategies to overcome them. This knowledge provides a view to re-visit current strategies used to develop new cryopreservation protocols as well as to improve current cryopreservation methods, in order to progress towards a more robust cryopreservation strategy which regards both the cell system being processed and its final application.

Resumo

A crescente necessidade de productos biológicos complexos para aplicações terapêuticas, ensaios farmacológicos e toxicológicos e investigação científica fundamental exige a produção de um grande número de amostras devidamente caracterizadas. Tal implica o desenvolvimento de metodologias de criopreservação efectivas que sejam capazes de lidar com o aumento de escala e a automatização do processo e reflectir as propriedades físicas e biológicas das células já que estas podem ser profundamente alteradas durante o processo. Actualmente, estão estabelecidos métodos eficientes de criopreservação para uma vasta variedade de tipos de células, as quais podem ser congeladas como suspensões de células isoladas com elevadas taxas de sobrevivência. No entanto, estes métodos não são suficientes para garantir a retenção de elevados níveis de recuperação em sistemas celulares complexos tais como culturas celulares em monocamada e agregados tri-dimensionais, tecidos ou órgãos, ou sistemas mais sensíveis tais como culturas celulares primárias ou células estaminais embrionárias humanas.

O principal objectivo desta tese é o desenvolvimento e validação de protocolos de criopreservação consistentes, eficientes e compatíveis com as boas práticas de manufactura (“GMP”), para sistemas celulares complexos, os quais permitam o redimensionamento e automatização do processo e que sejam capazes de lidar com elevado número de amostras bem caracterizadas para investigação, terapia e/ou aplicações farmacêuticas. Especificamente, foram aqui desenvolvidas estratégias de criopreservação para monocamadas bi-dimensionais (2-D) funcionais e agregados tri-dimensionais (3-D) de culturas primárias, sendo o objectivo final o desenvolvimento de protocolos de criopreservação optimizados para células estaminais embrionárias humanas. O racional por detrás da selecção dos modelos celulares 2-D e 3-D foi baseado nas características importantes que

estes sistemas partilham com as colónias de células estaminais embrionárias humanas, nomeadamente a manutenção da função específica e as interações célula-célula e célula-matriz como principais requisitos para uma criopreservação bem sucedida. Para alcançar este objectivo, foi desenvolvida uma abordagem integrada através da manipulação de passos específicos dentro do fluxo de criopreservação geral, nomeadamente condições de pré-congelamento, método de congelamento e condições de recuperação após o descongelamento.

No **Capítulo I**, é apresentada uma introdução geral ao tema da criopreservação, dando particular relevância ao impacto dos diferentes parâmetros dentro do fluxo de criopreservação na recuperação das células após o descongelamento. É dada especial atenção ao estado da arte relativamente às limitações actuais desta tecnologia quando aplicada a diferentes sistemas celulares, nomeadamente sistemas 2-D e 3-D e células estaminais embrionárias humanas, bem como estratégias propostas para ultrapassar essas limitações.

No **Capítulo II**, foi validada uma nova estratégia para a criopreservação de células em suspensão baseada em miniaturização e automatização, a qual é da maior importância no estabelecimento de bancos de células para um elevado número de amostras. Para tal, estudou-se o efeito das condições de pré-congelamento e congelamento na viabilidade pós-descongelamento de células do adenocarcinoma do cólon, Caco-2, quando congeladas como suspensões celulares. Nomeadamente, avaliou-se a redução do volume do recipiente de congelamento, dos tradicionais 1 ml de um criofrasco para os 25 µl dos micro-criosubstratos testados, e a escolha e método de adição do crioprotector testado. Por forma a determinar os parâmetros importantes que devem ser investigados após o descongelamento, diferentes propriedades celulares foram avaliadas, tais como as capacidades de proliferação e diferenciação celular. Os resultados demonstraram que a redução do criorecipiente não afectou a integridade da

membrana celular após o descongelamento nem a função ou o potencial de diferenciação, o que é um requisito fundamental para possíveis utilizações terapêuticas.

Nos **Capítulos III e IV**, foi investigada a criopreservação de sistemas 2-D e 3-D com uma vasta variedade de aplicações em ensaios pré-clínicos, tendo em conta os desafios específicos de cada sistema. Alginato ultra-viscoso (UHV) de qualidade clínica foi utilizado para a encapsulação das células antes da criopreservação como principal estratégia para evitar a descolagem da monocamada, a desintegração das colónias e a perda de contacto célula-célula e célula-matriz e também devido ao seu potencial para ser utilizado como uma matriz de qualidade clínica para terapia celular. De forma a evitar o atraso na morte celular observado para estes sistemas e com o objectivo de desenvolver um protocolo de criopreservação compatível com práticas “GMP”, uma solução comercial livre de proteínas e de soro e suplementada com inibidores de apoptose, CryoStorTM, foi comparada com o meio de cultura como solução veículo para o crioprotector. No **Capítulo III**, foi investigada a criopreservação de células aderentes à superfície de poços de placas de cultura. Duas linhas celulares, neuroblastoma N2a e adenocarcinoma do colon Caco-2, foram utilizadas devido às suas distintas características estruturais e funcionais. A combinação da encapsulação em alginato UHV e a utilização da solução CryoStorTM para o congelamento desta duas linhas celulares a velocidade lenta resulta em respostas celulares específicas que podem levar à optimização de protocolos para monocamadas celulares, tal como demonstrado para as células N2a. Apesar de ter sido observado que a encapsulação em alginato UHV aumenta a recuperação celular através da redução de danos na membrana plasmática e descolagem das células, a utilização da solução CryoStorTM foi crucial para a total recuperação da actividade metabólica e capacidade de diferenciação em 24 horas após descongelamento; neste caso, a encapsulação debaixo de uma camada de alginato não conferiu protecção

adicional às células Caco-2 mas foi crucial para a manutenção da aderência e integridade das redes neuronais de células N2a diferenciadas.

O **Capítulo IV** descreve o desenvolvimento de uma estratégia integrada que permite a cultura por tempo prolongado e a criopreservação de culturas primárias de neuroesferas do cérebro com alta viabilidade, reduzindo tanto o tempo de recuperação após o descongelamento como, significativamente, a fragmentação das neuroesferas. O processo de encapsulação em alginato UHV não afecta o metabolismo celular central, nem a diferenciação celular ou extensão das células em redes usualmente observadas entre neurónios-astrócitos dentro da estrutura da neuroesfera. Além disso, a utilização da solução CryoStor™ fornece protecção adicional para agregados não-encapsulados e encapsulados quando comparada com o meio de cultura suplementado com soro como meio de criopreservação. Os resultados obtidos com os dois modelos demonstraram que a encapsulação em alginato UHV e a utilização da solução de CryoStor™ são abordagens excelentes que visivelmente melhoraram a sobrevivência dos sistemas celulares 2-D e 3-D, permitindo a reduzindo os danos na membrana plasmática e aumentando a integridade de toda a monocamada/agregado, bem como a retenção da actividade metabólica e função celular específica/estado de diferenciação após o descongelamento. Estas, como mencionado, são características obrigatórias para aplicações em investigação pré-clínica e terapia celular.

No **Capítulo V**, os passos de criopreservação estudados e optimizados nos capítulos III e IV foram combinados com o objectivo de desenvolver uma estratégia de criopreservação integrada para células estaminais embrionárias humanas. Foi demonstrado que, quando aplicados a células estaminais embrionárias humanas, os protocolos de velocidade lenta de congelamento optimizados para os modelos celulares 2-D e 3-D não são adequados para a criopreservação eficiente das colónias. Assim, diferentes abordagens foram

avaliadas, nomeadamente a vitrificação em detrimento de uma velocidade lenta de congelamento. A vitrificação de colónias aderentes resultou em taxas de recuperação das colónias significativamente mais elevadas quando comparadas com a abordagem de velocidade lenta de congelamento: elevada sobrevivência e reduzida fragmentação das colónias foram observadas imediatamente após o descongelamento e durante a continuação da cultura até à passagem celular seguinte. Este protocolo resultou em baixas taxas de diferenciação e manutenção da pluripotência após o descongelamento. Este novo método de vitrificação baseado em superfícies facilita o desenvolvimento de um processo de criopreservação em larga escala e com elevado rendimento para células estaminais embrionárias humanas que reduza o tempo necessário para amplificar as amostras congeladas e assim suportar a utilização generalizada destas células em investigação, terapia ou aplicações farmacológicas.

O **Capítulo V** consiste numa discussão geral, onde as perspectivas futuras e as principais conclusões do trabalho são apresentadas.

Esta tese contribui substancialmente para o estabelecimento de metodologias de criopreservação efectivas para sistemas celulares complexos, nomeadamente monocamadas 2-D, agregados 3-D e colónias de células estaminias embrionárias humanas. A identificação e análise de pontos críticos no desenvolvimento do processo resultou em propostas válidas para estratégias efectivas na resolução destas limitações. O conhecimento adquirido fornece uma forma de rever as estratégias actualmente utilizadas para desenvolver novos protocolos de criopreservação bem como melhorar os procedimentos, por forma a progredir no sentido de uma estratégia de criopreservação mais robusta que tenha em atenção o sistema celular a ser processado bem com a sua aplicação final.

Thesis publications

1. **R. Malpique**, A. Katsen-Globa, M. J. T. Carrondo, H. Zimmermann, P. M. Alves. “Cryopreservation in Micro-Volumes: Impact upon Caco-2 Human Colon Adenocarcinoma Cells Viability, Proliferation and Differentiation”, *Biotechnology & Bioengineering* 98 (1): 155-66 (2007).
2. **R. Malpique**, Friederike Ehrhart, Alisa Katsen-Globa, Heiko Zimmermann, Paula M. Alves, “Cryopreservation of Adherent Cells: Strategies to Improve Cell Viability and Function After Thawing”, *Tissue Engineering Part C: Methods* 15(3): 373-386 (2009).
3. **R. Malpique**, L. Osório, D. Ferreira, F. Ehrhart, C. Brito, H. Zimmermann, P. M. Alves. “Alginate encapsulation as a Novel Strategy for the Cryopreservation of Neurospheres”, *Tissue Engineering, ahead of print*;
4. **R. Malpique**, M. Serra, C. Brito, H. Zimmermann, P. M. Alves. “Novel Strategies towards High-throughput Cryopreservation of Human Embryonic Stem Cells”, submitted to *Stem Cells*.

Abbreviations

Abbreviation	Full form
ASMA	α -smooth muscle actin
bFGF	basic fibroblast growth factor
BME	basal medium Eagle's
Caco-2	human colon adenocarcinoma cell line
DMEM	Dulbecco's minimum essential medium
DMSO	dimethyl sulfoxide
EB	embryoid bodies
ECM	extracellular matrix
EM	electron microscopy
EB	ethidium bromide
EG	ethylene glycol
FBS	fetal bovine serum
FDA	fluoresceine diacetate
FOXA2	forkheadbox A2
GFAP	glial fibrillary acidic protein
Glc	glucose
Gln	glutamine

Abbreviation	Full form
HSA	human serum albumin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HES	hydroxyethyl starch
hESC	human embryonic stem cells
hFF	human foreskin fibroblasts
IgG	immunoglobulin G
IgM	immunoglobulin M
IIF	intracellular ice formation
Lac	lactate
LN ₂	liquid nitrogen
MAP	microtubule-associated protein
mESC	mouse embryonic stem cells
N2a	mouse neuroblastoma cell line
NF-L	low-molecular weight neurofilament protein
PBS	phosphate buffered saline
PFA	paraformaldehyde
Oct-4	transcription factor octamer-4
OPS	open pulled straw

Abbreviation	Full form
RA	retinoic acid
SCED	single cell enzymatic dissociation
SSEA-1	stage-specific embryonic antigen-1
SSEA-4	stage-specific embryonic antigen-4
T _h	homogeneous nucleation temperature
T _m	equilibrium melting temperature
T _g	glass transition temperature
TRA-1-60	tumour rejection antigen-1-60
TX-100	Triton X-100
UHV	ultra-high viscous

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1 CONCEPTS IN CRYOPRESERVATION

Cryopreservation is traditionally defined as the maintenance of biologics at temperatures typically below the glass transition of pure water (-132°C), at which biological metabolism is dramatically diminished (Baust et al. 2006).

The science of cryobiology, from which cryopreservation methodologies have developed, began in the 1940s with the publication of Basil Luyet's "Life and Death at Low Temperature" and the discovery by Polge and colleagues of glycerol's effectiveness as a cryoprotective agent (CPA) for fowl spermatozoa (Polge et al. 1949). Cryobiology evolved in subsequent years with Lovelock's proposal in 1953 of cell damage during freezing through denaturation by concentrated salts (Lovelock 1953) and Lovelock and Bishop's discovery in 1959 of dimethyl sulfoxide (DMSO) as a cryoprotective agent based on rational predictions (Lovelock et al. 1959). Mazur's "Two-Factor Hypothesis", which relates survival of cells in solution to a dependence on the rate of cooling, was a landmark event to which current cryopreservation methodologies are closely linked (Mazur 1965), although the precise mechanism of slow-freezing injury remained unsolved.

Nowadays, cryopreservation provides unique benefits for many medical and industrial applications including blood transfusion, bone marrow transplantation, artificial insemination, *in vitro* fertilization, pharmacology testing or food storage as well as for fundamental scientific research by allowing long-term preservation of laboratory samples and their transfer between research institutes. Major advantages of cryopreservation applications for mammalian cells and tissues in the clinical field include the possibility of banking a large quantity of samples for regenerative and reparative medicine applications and facilitating its transport between different medical centres.

Although effective cryopreservation methods are established for a wide variety of cell types, such currently used techniques may not be able to assure the retention of high degrees of recovery in complex systems such as tissues and

organs, 2-D monolayer cell cultures and 3-D aggregates, or more sensitive systems such as primary cultures or human embryonic stem cells.

1.1 METHODOLOGY OF CRYOPRESERVATION

A general description of the cryopreservation workflow for mammalian cells and tissues is schematized in Figure 1. Typically, it involves a pre-freezing treatment in which the cells are brought to the desired state at which they will be frozen (e.g. single-cell suspension, cell aggregates, adherent cell monolayers), the cell viability is evaluated and the cells are transferred into the cryovessel on which they will be frozen (e.g. vial, well-plate, straw). Before freezing begins, samples are loaded with a CPA (e.g. DMSO, glycerol) to help minimizing cryoinduced damage. The cells are then frozen at the desired cooling rate to the storage temperature at which they will be stored. Upon removal from storage, the samples are thawed, CPAs are removed from the sample by dilution and post-thaw cell recovery is evaluated.

Each step of the cryopreservation process is important and can have a strong influence on post-thaw recovery as will be discussed in detail in the following sections.

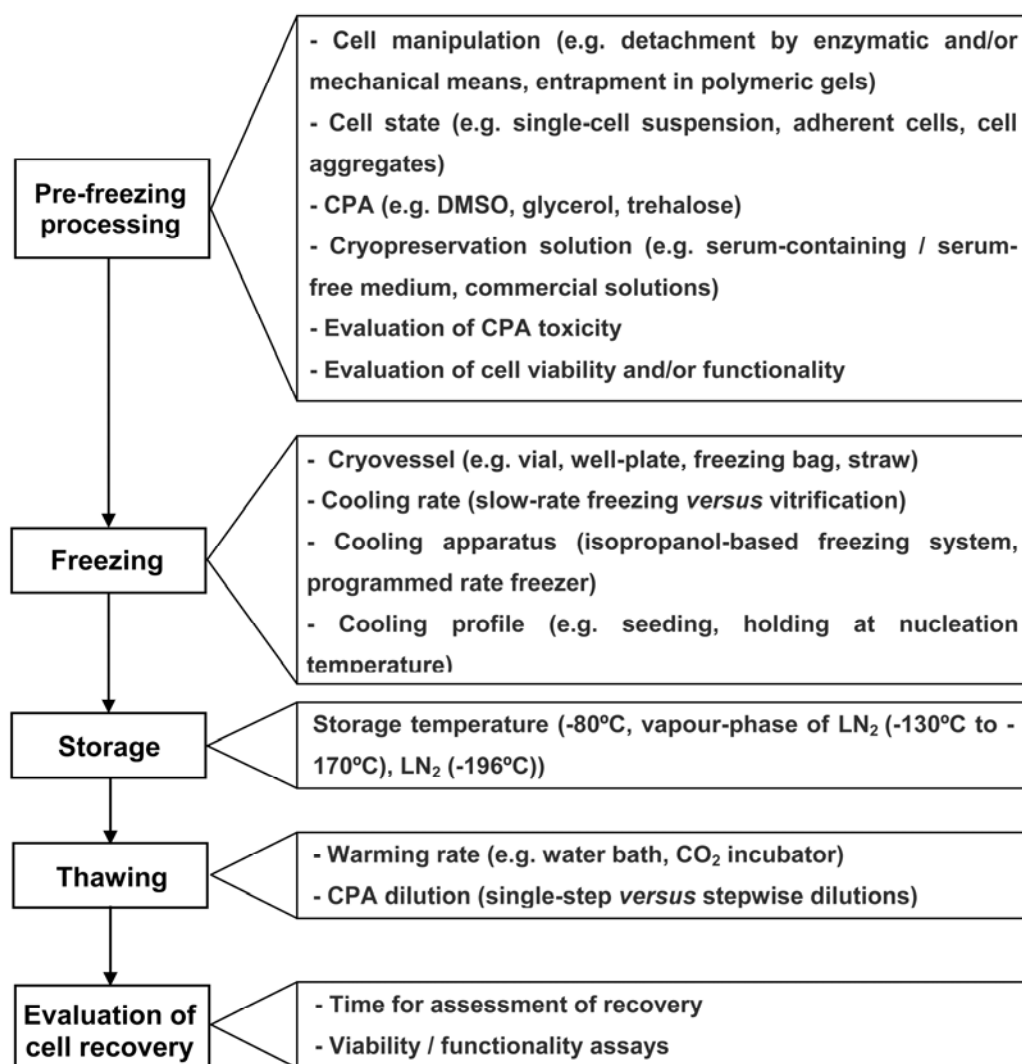


Figure 1. Schematic representation of the main steps composing the current routinely applied cryopreservation workflow for mammalian cells and tissues, with evidence given to the important parameters within each step.

1.1.1 Freezing: Mechanisms and Implications

Contrary to the usual impression, the challenge to cells during freezing is not their ability to endure storage at ultra-low temperatures; rather it is the lethality of an intermediate zone of temperature (~-15°C to -60°C) that a cell must transverse twice - once during cooling and once during warming. Once a sample reaches the temperature of the vapour phase of liquid nitrogen (LN₂) (<-130°C) no

thermally driven reactions occur in the aqueous system as liquid water does not exist below such temperatures. The only physical states that do exist are crystalline or glassy, and in both states the viscosity is so high ($>10^{13}$ poises) that diffusion is insignificant over less than geological time spans.

The conservative methods used for cryopreservation of cells and tissues are based on the slow-rate freezing approach, which consists in freezing the samples in the presence of a CPA at a selected cooling rate to minimize the probability of ice formation inside the cells. The slow cooling rate allows the cells to dehydrate by maintaining equilibrium with the partially frozen extracellular solution (Mazur 1963).

A diametrically different approach to cryopreservation is vitrification. In vitrification procedures there is an attempt to prevent ice formation throughout the entire sample by applying extremely high cooling rates ($>10^4$ °C/min) together with high concentrations of CPAs (6 - 8M) (Fahy et al. 1984).

A comparison of these two approaches, which will be analysed in detail in sections 1.1.1.1 and 1.1.1.3., is summarized in Table I.

Table I. A comparison of the two most common approaches to cryopreservation (adapted from Paynter (Paynter 2008)).

Cryopreservation Step	Slow-rate Freezing	Vitrification
Exposure to CPA	<ul style="list-style-type: none"> - Cells equilibrated with 1–2 M of permeating CPA; - Stepwise addition will reduce osmotic stress; - Duration and temperature of steps must be controlled. 	<ul style="list-style-type: none"> - Cells exposed to high concentrations (6–8 M) of CPA; - Full equilibration with CPA is not necessary; - CPA can be permeating, non-permeating or a mixture of the two; - Having achieved partial equilibration with lower CPA concentrations, short exposure to high concentrations will result in removal of water from the cells; - Duration and temperature of CPA loading and removal steps must be controlled.
Cryovessel	<ul style="list-style-type: none"> - Vials, straws, bags, well-plates; - Large capacity accepted. 	<ul style="list-style-type: none"> - Straws, loops; - Small volume is essential.
Cooling rate	<ul style="list-style-type: none"> - Most commonly 1°C/min; - Freezing can be achieved using controlled rate freezer or simpler device (e.g. isopropanol-based system); - Seeding ice formation should be performed to ensure linear cooling rate. 	<ul style="list-style-type: none"> - Freezing should be as rapidly as possible (cooling rates $> 10^4$ °C/min); - Usually achieved by direct exposure to LN₂.
Storage	<ul style="list-style-type: none"> - Long-term storage at less than -130°C; - Samples can be stored at higher temperatures (e.g. -80°C) for shorter periods. 	<ul style="list-style-type: none"> - Samples must be stored below -140°C to prevent ice crystal growth.
Warming rate	<ul style="list-style-type: none"> - Sample must be warmed as rapidly as possible to avoid recrystallisation of intracellular ice nuclei. 	<ul style="list-style-type: none"> - Sample must be warmed as rapidly as possible to avoid ice cristallisation.

1.1.1.1 Slow-rate Freezing

Due to the large fraction of water in biological cells and tissues, the phase transition of water to ice is of critical importance to the field of cryobiology. Below the equilibrium melting point, water will become thermodynamically unstable and will exist in a metastable or supercooled state (Debenedetti et al. 2003). On the other hand, although the thermodynamic freezing point (the highest temperature at which ice can coexist with the cytoplasmic milieu) of most cells is -0.5°C , cells and their surrounding medium remain unfrozen down to $\sim -5^{\circ}\text{C}$ both due to supercooling and because of the depression of the freezing point by the protective solutes that are frequently present. Between -5 and $\sim -15^{\circ}\text{C}$, ice forms in the external medium either spontaneously, due to breakdown of the metastable state of the supercooled solution (homogeneous nucleation) or as a result of seeding the solution with an ice crystal (heterogeneous nucleation), but the cell contents remain unfrozen and supercooled, presumably because the plasma membrane (PM) blocks the growth of ice crystals inside the cytoplasm.

At present, there are three dominant hypotheses that attempt to explain the mechanism by which extracellular ice interacts with the PM to initiate intracellular ice formation (IIF): (i) the “Protein-Pore Theory” of Mazur (Mazur 1965), which hypothesize that external ice could seed the supercooled cytoplasm by growing through aqueous pores in the PM; (ii) the theory of cytoplasm nucleation by external ice due to disruption of the PM (Steponkus 1984), which may be a result of a critical osmotic pressure gradient across the PM (Muldrew and MGann 1990); (iii) the theory of surface-catalyzed heterogeneous nucleation (Toner 1990), according to which the external ice can induce the formation of intracellular ice without physical disrupting the integrity of the PM, which behaves as a nucleation site for internal ice when acted on by extracellular ice. Although each of these theories proposes an alternative means by which ice can enter the cell, each one supports the assertion that extracellular ice phase plays a

major role in the biophysical response of cells to the cryopreservation process as it interacts with the PM to induce the formation of intracellular ice (Mazur 1965; Toner et al. 1993).

As temperature decreases, the extracellular solution becomes increasingly concentrated in solutes, which results in a chemical potential imbalance between the cytosol and the unfrozen external solution. In response to this difference in potential, water flows out of the cell and freezes externally (Mazur 1984). The rate of water efflux (via osmosis through the PM) is proportional to the magnitude of the osmotic pressure difference across the PM and the permeability of the PM to water (Mazur 1984), which may vary considerably between different cell types, thus leading to different responses of various cells to the same freezing protocol (Toner et al. 1993). The subsequent physical events in the cell depend on cooling velocity (Mazur et al. 1972) and will be discussed in section 1.2.1.

1.1.1.2 Cryoprotective Agents (CPAs)

CPAs are added to the cryopreservation medium, typically an isotonic cell culture or buffered electrolyte medium, to help reducing the injury to cells during freezing and thawing. These molecules are usually separated into two broad classes based on their ability to diffuse across the cell PM: (i) penetrating CPAs, which are able to move across the PM (e.g. DMSO, glycerol and 1,2-propanediol); and (ii) non-penetrating CPAs, such as hydroxyethyl starch, sucrose and other sugars, which cannot enter the cells. Classification of CPAs is summarized in Table II.

Table II. Classification of CPAs by their molecular weights and other properties (adapted from Kuleshova *et al.* (Kuleshova et al. 2007)).

Penetrating CPAs	Non-penetrating CPAs	
Low-molecular weight agents (MW < 100 Da)	Sugars (180 < MW < 594 Da)	High-molecular weight agents (MW > 1000 Da)
Polyalcohols Ethylene Glycol (EG) DMSO Propylene glycol Glycerol Butandiol: 1,2-/2,3-butandiol Formamide, acetamide	Monosaccharides: fructose, glucose, lactose, maltose Disaccharides: sucrose, trehalose Polysaccharides: raffinose	Ficoll Dextran Polyvinyl pyrrolidone Polyethylene glycol Polyvinyl alcohol (PVA) PVA-derived ice blocking agents Hydroxyethyl starch (HES)

The exact mechanisms by which penetrating CPAs are able to protect cells from cryoinduced injury are not fully understood. They have been attributed to both their non-specific colligative ability to reduce the concentration of damaging solutes and to solute-specific changes in solution properties and interactions with biomolecules. Lovelock was the first to propose that penetrating CPAs colligatively reduce the concentration of damaging electrolytes at a given subzero temperature (Lovelock 1953a; Lovelock 1953b). Mazur and colleagues proposed that penetrating CPAs increase the unfrozen intra- and extracellular fraction, thereby reducing the physical and biochemical effects that occur due to the close contact of the cells concentrated in the unfrozen fraction (Mazur et al. 1985; Ishiguro et al. 1994), interaction of the cells with the encroaching ice, and/or damaging concentrations of intracellular ice (Levitt 1962). Alternatively, Pegg and Diaper have suggested that penetrating CPAs function to reduce the extent of the cell volume change during slow-rate freezing and thawing (Pegg et al. 1989; Pegg et al. 1991). Another theory ascribes protection from CPA to solute-specific interactions with PM proteins or phospholipids bilayers that lead to the stabilization of the PM (Anchordoguy et al. 1987; Muldrew et al. 2004).

Non-penetrating CPAs are generally relatively high molecular weight, long-chain polymers that are soluble in water and can only be taken up by cells through endocytosis or induced processes. They are thought to act by dehydrating the cells before freezing, thereby reducing the amount of water that the cell needs to lose to remain close to the osmotic equilibrium during freezing. The cytoplasm does not supercool to the same extent, and therefore intracellular ice becomes less likely at a given cooling rate (Muldrew et al. 2004). When high concentrations of these CPAs are combined with rapid cooling their primary mechanism of action appears to be the induction of vitrification by forming a glassy material at the surface of the cell structure, which helps to stabilize the PM and proteins (Rall et al. 1985).

The ability of trehalose and other polysaccharides to protect membranes and proteins against the destructive effects of dehydration by serving as a substitute for structural water associated with their surface has also been described and provided a basis for the water replacement hypothesis (Crowe et al. 1973; Clegg et al. 1982), which offers a good explanation for at least part of the mechanisms underlying dehydration tolerance *in vivo*.

Survival of cells subjected to cryopreservation depends not only on the concentration of CPAs but also on cells permeability to the select CPA and on the time of incubation. This is usually empirically determined and commonly lasts from 10 to 30 minutes, and is carried out at temperatures close to 0°C (usually 4°C) to minimize the possible cytotoxic effect.

1.1.1.3 Vitrification

Vitrification is defined as glass-like solidification and/or complete avoidance of crystal formation during cooling and warming. This state is achieved in systems that are sufficiently concentrated and/or that are cooled sufficiently rapidly so that the increase in viscosity inhibits molecular rearrangement into a crystalline pattern. As cooling progresses, viscosity increases to the point at which

translational molecular motion is essentially halted and the solution becomes a glass. The resultant solid retains the random molecular arrangement of a liquid but has the mechanic properties of a solid (Taylor et al. 2004).

Vitrification is typically achieved by increasing the concentration of non-penetrating CPAs that interact strongly with water and therefore prevent water molecules from interacting to form ice, together with applying ultra-high cooling rates ($> 10^4$ °C/min) through the temperature region of potential crystallisation to reach the amorphous glassy state before ice crystals have the opportunity to form (Rall et al. 1985; Taylor et al. 2004). In this case, the samples go through directly from a liquid phase to a glassy state without suffering any nucleation as shown in Figure 2. The equilibrium melting temperature of water (T_m), the temperature of water which contains no nucleating agents, i.e. the homogeneous nucleation temperature (T_h), and the glass transition temperature (T_g) are all lowered by the addition of a CPA. If enough CPA is added, the freezing temperature T_h becomes so low that it reaches T_g , the temperature at which the water-CPA mixture forms a glass (vitrifies) rather than freezes into ice crystals (Fahy et al. 1984).

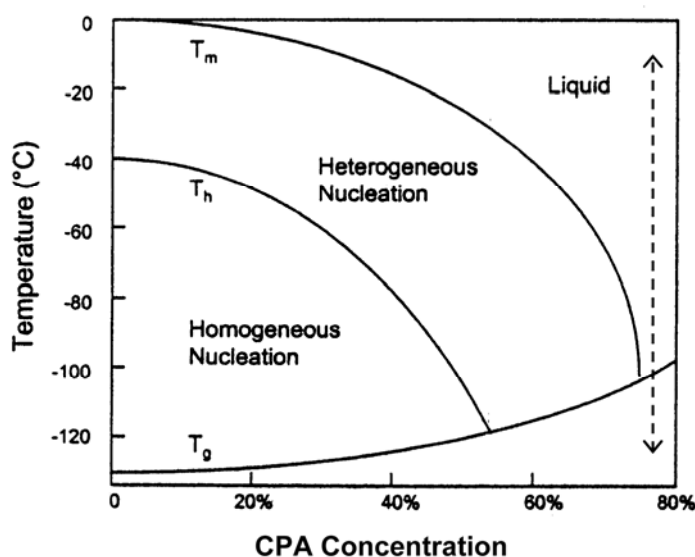


Figure 2. Typical binary phase diagram for an aqueous mixture of a CPA showing the principal events and phase changes associated with cooling and heating. (adapted from Fahy *et al.* (Fahy et al. 1984)).

This method minimizes cell exposure to non-physiological temperatures and circumvents problems associated with ice formation, cell dehydration, and the control of cooling rates. However, the use of ultra-high cooling rates produces a metastable state that can lead to devitrification (and ice crystallisation) on rewarming. Thus, the warming process needs to be rapid enough to avoid ice crystal formation.

Vitrification has been shown to provide effective preservation for a number of cells and tissues that have been difficult to cryopreserve by slow-rate freezing protocols, including monocytes, ova, early embryos and pancreatic islets (Rall et al. 1985; Takahashi et al. 1986; Vajta et al. 1998; Taylor et al. 2004).

1.2 BIOLOGICAL EFFECTS OF ICE FORMATION - FREEZING DAMAGE

There is an apparent contradiction between the concept of cryopreservation and experimental findings that living cells are often damaged by the cryopreservation process itself.

During the freezing process, when the environmental temperature drops from 37°C to near -196°C, cells are subjected to a range of thermal, chemical and mechanical forces that can profoundly affect their biological function and might lead to the occurrence of different potentially lethal phenomena to the cells.

The fundamental basis of all biologic and chemical processes is molecular activity and mobility, which are governed by thermal energy. This means that, as temperature is lowered, molecular motion is slowed, which slows down chemical reactions, including the biochemical processes that constitute metabolic activity, and biophysical processes, such as diffusion of ions and osmosis. As temperature decreases further, the rate of biophysical processes becomes significantly important, especially at subzero temperatures when phase changes lead to both ice formation and solute concentration changes (Taylor 1984; Taylor et al. 1987).

As ice forms in the extracellular solution, the electrolyte concentration increases by several orders of magnitude relative to isotonic conditions with consequent loss of cell water, which may lead to severe cell dehydration and extensive volume excursions. Moreover, cells may be mechanically deformed by ice crystals formed in the extracellular solution and ice may form inside the cells disrupting intracellular structures (Mazur 1965; Mazur 1970). Rupture of the PM may occur due to the simple mechanical action of advancing ice crystals, the osmotically driven water efflux caused by the increased osmotic pressure of the extracellular solution (Muldrew et al. 1994) or the large potential difference across the cells caused by the transient electric field associated with an advancing ice front in a weak electrolyte solution (Steponkus 1985).

Even when extra- and intracellular ice formation are not directly implicated in cell damage, the sensitivity of biological structures to decreased temperature is well documented. There are well described examples of cold denaturation involving the spontaneous unfolding of proteins or dissociation of the multi-subunit structure into biological inactive species, which may or may not reassemble on rewarming to normal temperatures (Brandts et al. 1970; Dixon et al. 1981). PM components are also affected by cooling, such that the PM properties related to both the selective diffusion barrier to solutes and active regulation involving PM-associated proteins (e.g. ion transporters) as cooling affects the thermophysical properties of PM lipids. During cooling, PM phospholipids undergo an abrupt change from a disordered fluid, or liquid crystalline state, to a highly ordered hexagonal lattice (gel state) at a specific transition temperature, and possibly to a phase separation within the plane of the PM. Such cold-induced changes in the degree of PM fluidity render it thermodynamically unfavourable for membrane proteins to remain in the gel phase, which may lead to a change in permeability and alteration in the solute barrier function of the PM (Quinn 1985). In addition to phase separations another form of cold-induced damage to the PM includes the actual loss of phospholipids,

which is intuitively more deleterious than phase changes that may largely be reversible.

The extent of cryoinduced damage to mammalian cells and thus the effectiveness of the cryopreservation process is affected by several factors, including cell type and size, growth phase, growth medium composition, cell water and lipid contents, cell state and density at freezing (e.g. single-cells in suspension *versus* adherent monolayers), composition of the cryomedium, cooling rate, temperature and duration of storage, warming rate and recovery medium (Frederickx et al. 2004).

Due to the complexity of biological systems, the exact mechanisms of cell damage during cryopreservation have not yet been elucidated. Nevertheless, the possible contributions of the different cryopreservation parameters to the cryoinduced cell damage and the different modes of death due to freezing injury have been reported and will be discussed in the following sections.

1.2.1 Effect of the Cryopreservation Parameters on Cell Injury

1.2.1.1 Cryoinjury Associated with the Pre-freezing Processing

1.2.1.1.1 Cell Manipulation

Prior to cryopreservation cells may be subjected to a multitude of processes, such as genetic modification, selection of subpopulations or enzymatic or mechanical detachment from the culture surface, which can non-lethally stress the cells (shear stresses, nutrient/oxygen deprivation, shifts in the PM composition), but may compromise the ability of cells to survive the stresses induced by the cryopreservation process. Thus, pre-freezing processing should be evaluated for its impact on post-thaw cell viability. Monitoring cells for early signs of apoptosis or shift in metabolism to stress pathways may also be helpful in determining if a pre-freezing protocol may be potentially harmful to the cells.

1.2.1.1.2 Cell State

Cryopreservation at temperatures below -130°C using 5% to 20% DMSO in serum-supplemented culture medium and slow cooling rates is a routine procedure for the prolonged storage of many mammalian cell types, which has been carried out successfully for single cells or small cell clumps due to an increased understanding of the freezing response of isolated cells in suspension (Mazur 1965; Mazur 1984). However, cells which can be successfully cryopreserved as single-cells in suspension are generally severely damaged *in situ* during freezing (Pegg et al. 1979; Armitage et al. 1996). The differences between simple cell suspensions and structured, complex systems, such as cell monolayers, tissues or biosynthetic constructs, with respect to their responses to cooling, warming, and dehydration clearly impact their requirements for cryopreservation and preclude direct application of information from single-cell systems (Karlsson et al. 1996). In cell monolayers, such differences are mainly due to the presence of cell-cell and cell-matrix interactions, whereas in tissues additional factors are related to their fixed geometry and 3-D arrangement, which impose problems of heat and mass transfer, as well as the presence of different cell types with different permeability properties (Jacobsen et al. 1984; Pegg 2001).

Cryopreservation of Cell Monolayers (2-D Systems)

Several reports on post-thaw loss of viability and excessive cell detachment for different cell lines cryopreserved as monolayers (Hornung et al. 1996; Wusteman et al. 1997; Pasch et al. 1999; Ebertz et al. 2004) have been attributed to the presence of cell-cell and cell-matrix interactions, which have shown to render cells more sensitive to cryoinjury when compared to isolated cells in suspension as their breakdown would disrupt the structural and functional relationships between cells (Armitage et al. 1996; Acker et al. 1999). The close interactions between cells are potential sites for damage caused by the osmotic stresses and phase changes involved in the process of cryopreservation (Armitage

et al. 1995) and have been implicated in the differences in IIF kinetics found between cultured and isolated cells (Larese et al. 1992; Yarmush et al. 1992). There appears to be an enhanced susceptibility to IIF in cells adherent to a substrate when compared to cells in suspension, even in the absence of cell-cell interactions (Berger et al. 1996; Acker et al. 2001) although the exact relation between IIF and cryoinjury in adherent cells is still unclear (Armitage et al. 1996; Acker et al. 2002; Armitage et al. 2003). Finally, the extended morphology of attached cells may create conditions for cryopreservation-induced damage to the cell ultra-structure (cytoskeleton, focal adhesions or gap junctions) due to mechanical forces, which might be caused by extracellular ice or by the differential thermal contraction between cells and substrates (Liu et al. 2005; Liu et al. 2006).

Cryopreservation of 3-D Cell Systems

Despite the “routine” freezing of tissues being currently cryopreserved to meet clinical and research needs, including liver slices (Maas et al. 2000), heart valves and cartilage (Reardon et al. 1998), blood vessels (Farber et al. 2004), skin (Trent et al. 1998), and numerous engineered tissues (Baust et al. 1999), tissue quality is quite poor.

In 3-D multicellular systems (e.g. neurospheres, pancreatic islets or tissue biopsies) the different permeability and cryotolerance of the various cell types combined with heat and mass (water and CPAs) diffusion restrictions leads to differences in the freezing environment experienced by individual cells (Mazur et al. 1972; Karlsson et al. 1996; Ehrhart et al. 2009). Significant differences in the rate of water loss during freezing and CPA between the surface and interior of the tissue and uneven distribution of CPA within the tissue may result in a situation in which surface cells may need to be exposed to high, potentially toxic concentrations of CPA in order to attain the minimal necessary CPA concentration in the tissue interior. Concerning heat transport limitations, due to the

macroscopic size of the specimen and its finite thermal conductivity, there may be large thermal gradients from the surface to the interior, which imply non-uniform rates of cooling through the sample. Thus, optimal cooling and warming rates may not be achieved in large sections of the tissue (Diller 1992). Moreover, thermal gradients may induce mechanical stress due to uneven expansion and contraction in the biomaterial (Rubinsky et al. 1980; Diller 1992). Furthermore, ice crystals can intercalate the tissue and mechanically deform cells, or disrupt the intercellular junctions that are needed to maintain the functional 3-D tissue architecture of the aggregate (Karlsson et al. 1996). Finally, it has been hypothesized that due to cell-to-cell connexions in tissues, the transmission of cell stress and signals of stress response pathway leading to apoptosis can “flow” more freely between cells, thereby leading to increased cell death through signal amplification (Baust et al. 2006).

All of the factors mentioned above account for the loss of cell-specific function and/or metabolic activity, reduced cell number in comparison to unfrozen controls, disintegration and shape distortion reported for 3-D cell systems (Merchant et al. 1993; Purcell et al. 2003; Ehrhart et al. 2009).

1.2.1.1.3 Exposure to CPAs

The ideal CPA should interact favourably with cells and other biological materials, be non-toxic, protect during both freezing and drying, substitute for water, and have a high T_g (Ji et al. 2004). However, this is not true for many CPAs which can themselves cause damage to cells due to their toxicity or as a result of excessive osmotic forces during their addition and removal (Mazur et al. 1984; Fahy 1986).

Because CPAs typically enter and leave the cell at a rate slower than water, the initial response of a cell exposed to a CPA solution is to lose water by exosmosis. Likewise, a cell containing CPAs will initially swell when placed in an isotonic environment, as water enters the cell by osmosis. Thus, cells transiently

shrink when a CPA is added and then return to near-normal volume as the CPA permeates. During the removal of the CPA, cells undergo transient volume expansion, the magnitude of which depends on how the removal is effected and on the inherent permeability of the cell to water and CPA. In several cell types (e.g., oocytes), the addition of CPAs (DMSO and 1,2-propanediol) has been shown to cause chemical disruption of the cytoskeleton (Johnson et al. 1987; Joly et al. 1992). Even though the cell volume will eventually return to its isotonic value as the CPA permeates the PM and equilibrates, excessive volume excursions and the attendant high osmotic water fluxes can be deleterious. To avoid these, CPAs are usually gradually added and removed, changing the concentration of the extracellular solution in a stepwise fashion. However, the benefits of slow, stepwise CPA addition must be balanced against the deleterious effects of increased exposure times to the potentially toxic CPA. This can be reduced by decreasing the time or temperature of cell exposure to the CPA, or by using lower concentration. DMSO, the most commonly used cryoprotectant, has been associated with several systemic side-effects to patients after transplantation (e.g. nausea and hypertension) (Syme et al. 2004), as well as effects on cell cycle and apoptosis (Santos et al. 2003), and it is known to induce differentiation in embryonic stem cells (ESC) (Adler et al. 2006). Therefore, for medical approval it is of great importance that technology is available allowing the use of lower concentrations of DMSO or, if possible, its complete replacement by equally powerful, non-toxic CPAs. Chemicals such as glycerol and inositol, or the disaccharides sucrose and trehalose, enhance cell viability when combined with DMSO, or can replace it as a CPA for a variety of cell types (Mussauer et al. 2001; Ji et al. 2004).

1.2.1.2 Cryoinjury Associated with the Freezing Process

A major factor determining whether or not cells survive freezing to low subzero temperatures is the rate at which they are cooled. For slow-rate freezing

protocols, characteristic survival signatures generated by measuring cell survival as a function of cooling rate appear to be qualitatively similar for all cell types: cell survival is low at very low and very fast cooling rates, and there is an intermediate cooling rate yielding optimal survival. Such findings have been explained by Mazur's "Two-Factor Hypothesis" of freezing damage (Mazur et al. 1972), according to which there are two independent mechanisms of damage during freezing, one active at slow cooling rates, the other at fast cooling rates.

Typically, cryopreservation protocols are optimised by identifying the ideal cooling rate for a specific cell type that minimizes both dehydration and IIF. Commonly, plots of cell survival versus cooling rate take the form of an inverted U, as exemplified in Figure 3.

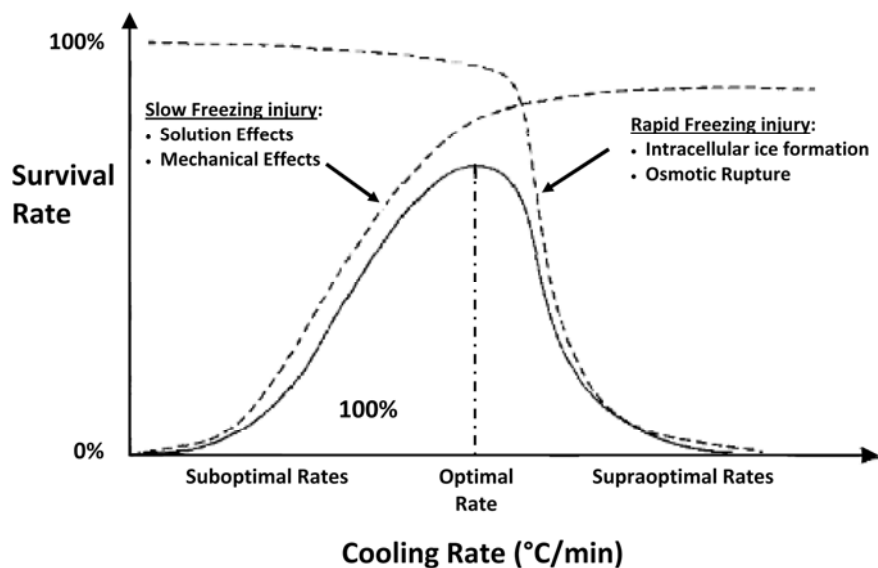


Figure 3. Survival signature of a hypothetical frozen-thawed cell, showing the competing modes of damage at slow and rapid rates of cooling, and the resulting optimal cooling rate (adapted from Karlsson *et al.* (Karlsson et al. 1996)).

Injury by Fast Cooling Rates

At fast cooling rates there is little to no transport (by exosmosis) of intracellular water out of the cell, the cytoplasmic supercooling increases rapidly

thus increasing the probability of intracellular ice nucleation. In this case, cell injury has been attributed to IIF (Mazur et al. 1972; Mazur 1984). Mechanical constraints due to the IIF (Mazur 1977) might cause damage to the PM (Fujikawa 1980), the membrane of intracellular organelles (Mazur 1966) or the cytoskeleton (e.g. microtubules) (Baust et al. 2006). Despite the strong evidence for involvement of IIF in cell damage during freezing, observed cases of innocuous ice formation in cells indicate that the presence of intracellular ice *per se* does not cause cell death, especially when the extension of intracellular crystallisation is limited (Mazur 1977; Rall et al. 1980; Karlsson et al. 1993).

Injury by Slow Cooling Rates

If the cooling rate is sufficiently slow, the cell will attempt to establish a state of equilibrium by expressing water through the PM, and high levels of dehydration ensue with concomitant increases in solute concentration, creating a state of extensive dehydration. Thus, at slow rates of cooling, cell injury is thought to be due to the effects of exposure to highly concentrated intra- and extracellular solutions or to mechanical interactions between cells and the extracellular ice. When ice forms in an aqueous solution, most solutes are excluded from the ice, and remain in a concentrated unfrozen solution. High concentrations of electrolytes and other solutes in the extracellular medium, which affect electrical or ionic interactions, and the resulting cell dehydration have been proposed as a source of cell damage – the so-called ‘solution effects’ (Lovelock 1953b; Lovelock 1957; Mazur et al. 1972). Other studies have focused on the potentially damaging effect of cell shrinkage as a response to a highly concentrated extracellular solution, and propose that water removal below a certain “minimum volume” results in irreversible changes and cell death in a variety of mammalian cells. (Meryman 1968; Meryman 1970; Steponkus et al. 1983).

Both mechanism of freezing injury are schematised on Figure 4.

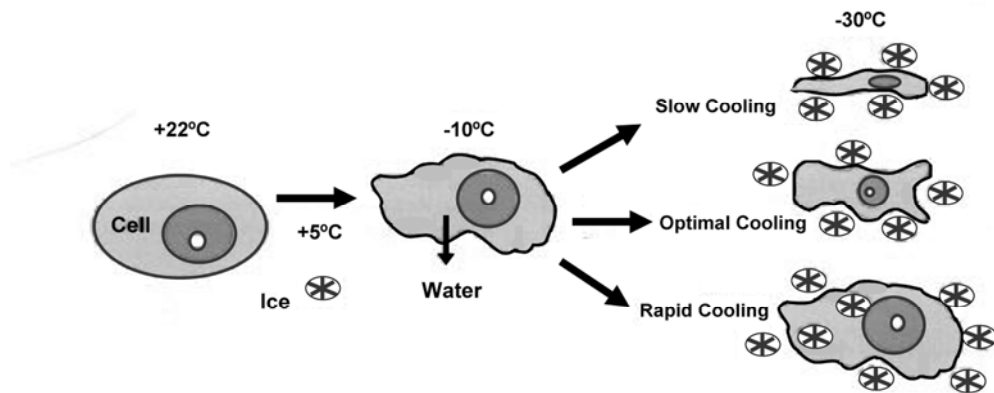


Figure 4. Schematic of physical events in cells during freezing (adapted from Mazur (Mazur 1984)).

Seeding of Extracellular Ice

When cryopreserving by a slow-rate freezing protocol, ice formation in the extracellular medium should be deliberately initiated by seeding at low degrees of supercooling. If ice formation is not induced by seeding, ice will form spontaneously when the solution is cooled sufficiently far below its equilibrium melting point. Because this process is stochastic in nature, ice formation will occur at random, unpredictable temperatures. Consequently, survival rates will be highly variable between repeated trials with the same freezing protocol. Furthermore, the extremely rapid crystallisation, which results when ice forms in a supercooled solution, can cause cell damage. Thus, the temperature at which ice forms has a profound influence on post-thaw viability (Toner et al. 1990; Karlsson et al. 1996).

To avoid the problems of uncontrolled ice formation in the extracellular solution, it is advisable to deliberately induce extracellular ice formation at temperatures slightly below the solution melting point. This can be achieved by touching the sample with a chilled needle or similar implement. Ice will form at the point of contact and spread through the biological sample (Karlsson et al. 1996). However, this procedure is not usually applied during routine cryopreservation practices in research and medical centres as it is not practical

and does not easily cope with commonly applied freezing devices, such as commercial isopropanol-based systems or programmable, control-rate freezers.

1.2.1.3 Cryoinjury during the Storage Period

Storage conditions will influence the shelf-life of a cryopreserved product. The highly concentrated, unfrozen solution that forms after the nucleation of the extracellular solution does not freeze completely until the system reaches the eutectic temperature, which varies depending on the composition of the cryopreservation solution. For a 10% DMSO solution, the eutectic temperature is approximately -70°C while other cryopreservation solutions commonly used have eutectic temperatures as low as -120°C . Storage of a product at or near the eutectic temperature implies that the extracellular solution is not fully solidified, and the cells will be surrounded by high concentration solutions which can in turn influence cell recovery (Shepard et al. 1976).

Frequently, for long storage periods, the temperature of LN_2 (-196°C) is needed, which is below the point of glass transition of pure water (near -140°C). At such a low temperature, there is insufficient thermal energy for chemical reactions, and the only deterioration that can occur in a biological sample is DNA damage by background radiation and cosmic rays (Mazur 1984). The shelf-life of cells stored at this temperature has been estimated to be of the order of 10^3 years (Mazur 1984). However, samples stored in LN_2 are subject to the risk of contamination with infectious agents (Tomlinson et al. 2000; Mazzilli et al. 2006). Thus, although temperatures may vary between -150°C to -180°C due to location within the freezer and LN_2 levels, storage at the vapour phase of LN_2 is the best choice for most situations as it offers the following benefits compared to immersion storage (Kenneth E. Avis 1999):

- (i) temperatures in the required range of -150°C to -180°C ;

- (ii) reduced risk of cryovessel contamination with infected LN₂;
- (iii) reduced risk of cryovessel explosion.

1.2.1.4 Cryoinjury Associated with the Warming Process

A cell that has survived cooling to low subzero temperatures is still challenged during warming, which can exert effects on survival comparable with those of cooling (Mazur 1984). During warming, viscosity falls, molecular motion becomes less slow, and water molecules may diffuse and rotate into the configurations required to nucleate ice, or to add to existing nuclei: small, innocuous intracellular ice particles that may have formed during freezing can coalesce into larger, damaging crystals during warming, by a process known as recrystallisation (Forsyth et al. 1986). The chance of large scale ice nucleation and/or growth occurring depends on the time the sample is exposed to lower viscosities while it is below the equilibrium freezing temperature. Recrystallisation is more likely to occur in rapidly cooled cells, because these will have a high degree of cytoplasmic supercooling and are more likely to contain intracellular ice nuclei. When cells are cooled slowly enough to preclude intracellular freezing, the response to warming rate is often highly dependent on the freezing conditions and cell type and is difficult to predict *a priori* (Karlsson 2001). For conventional freezing over the range of cooling rates used for most cell types (1-30°C/min), optimal warming rates should be as rapid as possible (>200°C/min) to minimize damage due to recrystallisation (Rall et al. 1984). However, in some cases rapid warming rates can yield lower post-thaw survival, especially for cells that have been frozen at a slow rate (Mazur 1984), which has been due to osmotic stresses that may occur during rapid rehydration of the cell.

1.3 CRYOPRESERVATION-INDUCED MODES OF CELL DEATH: NECROSIS VERSUS APOPTOSIS

Cryopreservation protocols are developed to reduce CPA toxicity and osmotic shock and provide optimal cooling rates in order to limit IIF and reduce

cellular volume excursions. Such protocols focus on overcoming freeze-induced cell death primarily due to IIF and chemo-osmotic stress, which results in PM disruption and subsequent necrosis (Paynter et al. 1997). This mode of cell death is characterised by the loss of PM integrity resulting in cell swelling and DNA cleavage in a random fashion.

Apoptosis has only recently been implicated as a contributing factor to cell death in a wide variety of cellular systems following cryopreservation (Baust et al. 2000; Schmidt-Mende et al. 2000; Baust et al. 2001; Yagi et al. 2001; Men et al. 2003; Villalba et al. 2004). This is a gene-regulated cell death mechanism characterized by cell shrinkage, PM blebbing, non-random DNA degradation, and ultimately phagocytosis by neighboring cells *in vivo* (Wyllie et al. 1980; Thornberry et al. 1998).

The cryopreservation-dependent signalling that induces apoptosis is unknown, although it is apparent that the activation of the apoptotic mechanism within the cells may be triggered as an indirect result of mild cryoinjury that is not directly lethal to the cells (i.e. damage to gap-junctions, extracellular matrix); or physical environmental stress encountered during cryopreservation as a direct result of the stress experienced during the freeze-thaw process (i.e. cold shock, osmotic stress). Therefore, the activation of apoptosis is likely to begin during cold storage, whereas the amplification and execution of the program may only be seen once returned to the energy-productive normothermic conditions. If a self-induced apoptotic mechanism is involved in cryoinduced damage, then a gradual loss of viability is expected as the apoptotic cascade needs time to activate and exert its effect on the cell after freeze-thaw.

Many of the factors which can initiate a cryopreservation-induced molecular-based mode of cell death response include metabolic uncoupling, production of free-radicals, alterations in PM structure and fluidity, deregulation of ionic balances, release of calcium from intracellular stores, osmotic fluxes, and CPA exposure (Baust et al. 2002; Baust 2005).

One possible “route” for cryoinduced apoptosis might be the physical stress that is exerted on the PM as a consequence of the “shrinkage and swelling” of a cell during the freeze-thaw process, which in combination with profound hyperosmolality may be activating death receptors on a cell’s surface resulting into onset of apoptosis (Green et al. 1998). Another possible means of apoptosis activation might be as a result of free-radical accumulation in the cytosol causing the activation of the mitochondrial permeability transition pore, which allows for the free diffusion of ions into and out of the mitochondria, thus disrupting its normal activity (Green et al. 1998). Finally, in response to probable sublethal cellular damage and DNA damage, the p53 gene may be activated, thereby initiating the apoptotic process within the cell (Evan et al. 1998). It is also possible that cryopreservation-induced apoptosis may not be linked directly to one particular apoptotic induction pathway but may be due to a combination of apoptotic initiation factors and pathways. More studies are necessary to determine which pathways are intimately linked. Figures 5 and 6 summarize the main contributions to cryoinduced cell damage that may occur during the cryopreservation workflow and the possible modes of death due to cryoinjury, respectively.

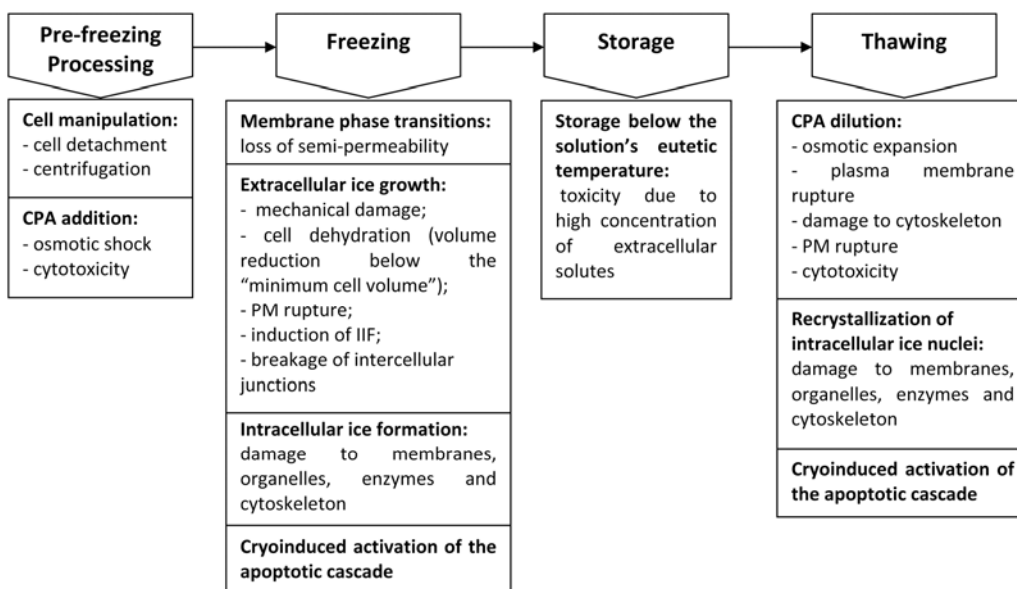


Figure 5. Summary of the main contributions to cryoinduced cell damage that may occur during the cryopreservation workflow.

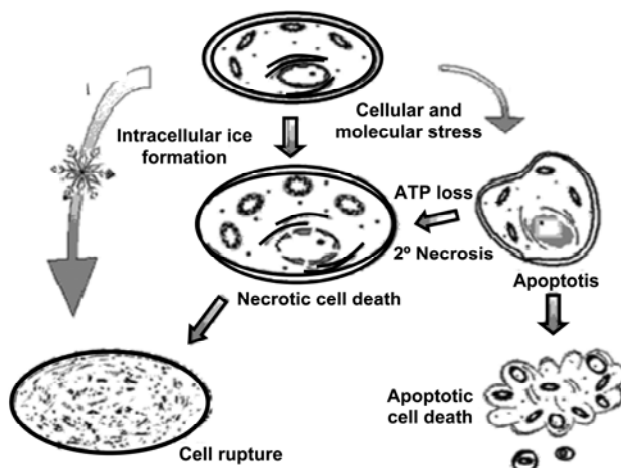


Figure 6. Modes of cryoinduced cell death (adapted from Baust et al. (Baust et al. 2006)).

1.4 EVALUATION OF POST-THAW RECOVERY

Although it is now well recognized that accurate and meaningful measures of post-thaw recovery are critical to the development of effective cryopreservation protocols, the lack of standardised and validated methods still makes data obtained from different laboratories seldom directly comparable.

Viability assays can be divided onto different categories: (i) physical PM integrity; (ii) metabolic activity; (iii) mechanical activity (attachment/contraction); (iv) mitotic activity (proliferation assays); (v) cell-specific function; and (vi) transplantation potential (Pegg 1989). Each of these assays provides important information and, typically, the use of one assay is not sufficient. For example, numerous studies have measured high levels of PM integrity for frozen and thawed hepatocytes but unless these cells attach to a surface and exhibit typically measured hepatocyte specific functions, the cells are not useful (Gomez-Lechon et al. 1984).

Studies have shown that the delayed molecular effects following thawing extend beyond that of cell death and may also lead to the loss of cell function, which can be substantial in homogeneous cell suspensions and increase as the system undergoing preservation becomes more complex (Paynter et al. 1995; Karlsson et al. 1996; Baust et al. 1999). A substantial post-thaw reduction in cell function has been documented in several cell types, such as hepatocytes (Guillouzo et al. 1999; Li et al. 1999), pancreatic islets (Rajotte et al. 1981), cardiac cells (Yokomuro et al. 2003), blood cells (Dannie 1996), and stem cells (Hunt et al. 2007).

It is also known that during the cryopreservation process cells experience the suppression of metabolic activity, and there can be a delay between thawing and the resumption of normal metabolic activity (Borel Rinkes et al. 1992). Furthermore, different studies have shown that short-term post-thaw assessment of cryopreservation outcome tends to yield overestimates due to sequential

apoptotic and necrotic processes contributing to cell death not evident immediately subsequent to thawing. Thus, recovery assays should be performed at proper post-thaw time points (Frim et al. 1978; Baust et al. 1998; Baust et al. 2002).

Methods such as the trypan blue exclusion assay and the lactate dehydrogenase (LDH) release assay, which are based on membrane disruption, have been commonly used in studying cryopreservation efficacy. Although the LDH assay is still used today as it can be used to both on single-cell monolayers as well as to assess whole tissue preservation, the most commonly set of cytotoxicity live/dead assays used nowadays are those that employ fluorescent indicator dyes (e.g. FDA, SYTO, ethidium bromide) as these allow the use multiwell-reading spectrofluorometers (Baust et al. 2006). However, these assays do not take in consideration that cells that have been frozen and thawed, and are still intact, have undergone extensive dehydration that may leave the PM transiently leaky, which may affect their permeation to commonly used viability indicators. Thus, metabolic assays, such as alamarBlue or MTT analysis have been preferred over membrane integrity indicators for a variety of cell and tissue types (Baust et al. 2006). Finally, as measurements on cell viability based only upon typical early stage event or metabolic indicators will not distinguish between apoptotic and living cells, apoptosis indicators have been used to assess cell recovery post-thawing, namely electron microscopy and DNA gel electrophoresis (Borderie et al. 1999; Baust et al. 2002; Baust et al. 2006).

Viability indicators must also be chosen according to the cell-system being cryopreserved as post-thaw evaluation of recovery for 3-D aggregates and tissues is far more complex than for single-cells. This is due to the lack of common standardized methods, the broad variety of aggregates systems with different functional properties (e.g. albumin secretion of hepatocytes and glucose levels for Langerhans' islets), and the difficulty in assessing the viability of each single cell

within the whole aggregate. Post-thaw dissociation is not a solution as cells may be damaged during enzymatic or mechanical destruction of the extracellular matrix and/or cell-cell contacts. Finally, such assays give no indication of survival of specific cell types and hence give no indication of the functionality of the tissue. Thus, measurement of cell metabolic activity, concerning major nutrient consumptions and metabolic consumption, as well as evaluation of cell-specific function, are often better choices for assessing cell/tissue recovery.

2 ALTERNATIVES TO THE TRADITIONAL CRYOPRESERVATION APPROACH

As stated above, the commonly applied cryopreservation protocols routinely used in research centres worldwide is far from optimal when there is the need for long term storage of more sensitive and complex systems for cell-based therapies and *in-vitro* pharmacological applications. The success of these applications depends, in part, on the ability of the cryopreservation process to maintain not just the structure but also the function of the cells in order to reduce time between cell storage and its use. With this purpose, optimized methodologies for the cryopreservation of such complex systems need to be developed, which take into account not only the properties of each cell type but also the specificity of the intended application (Karlsson et al. 1996; Acker et al. 2000). For clinical applications, such methodologies must be compatible with direct injection of the transformed biologic in comparatively large volumes of cryopreservation solution (Hernandez-Navarro et al. 1998). Thus, such applications would clearly benefit from a cryopreservation regime based upon trace/or low levels of CPA, or preferably a CPA-free preservation strategy, rather than the molar concentrations currently utilized, as well as the use of serum-free cryopreservation medium (Kawabe et al. 1990; Langer et al. 1993; Karlsson et al. 1996; De Loecker et al. 1997). Finally, limitations associated with the increased

costs and restricted capacity of the large standard storage volumes must be overcome.

Different cryopreservation methodologies alternative to the commonly applied protocols are reported in the literature; some refer to the use of structured substrates (Hornung et al. 1996), cell entrapment on biomaterials (Koebe et al. 1990; Zhou et al. 1997; Ji et al. 2004), the use of other CPAs less toxic than DMSO (Pasch et al. 1999; Ji et al. 2004; Ma et al. 2006) or the use of solutions specially designed to reduce the ionic and hydraulic imbalances induced in cells during hypothermia (Taylor et al. 1985; Taylor et al. 2001; Campbell et al. 2007). Such alternative methodologies will be analyzed in more detail in the following sections.

2.1 CELL ENTRAPMENT IN BIOMATERIALS

Previous studies have demonstrated that cell entrapment within a gel or polymeric matrix is a promising approach for the cryopreservation of 2-D and 3-D cell systems as cells are protected against mechanical damages during ice crystallisation and the danger of disrupting cell-cell contacts are reduced through immobilization within the hydrogel (Inaba et al. 1996; Zimmermann et al. 2007). Hepatocyte cryopreservation in alginate or collagen gels has been shown to provide enhanced viability when compared to cells cryopreserved in suspension (Birraux et al. 2002; Mahler et al. 2003). Cryopreservation of hESC adherent to a matrigel (gelatinous protein mixture secreted by mouse sarcoma cells) matrix allowed an increase on cell viability and reduced undesired cell differentiation (Ji et al. 2004). Algae-derived polysaccharides, such as agarose and alginate, have been reported as novel class of non-permeating (with respect to the cell) CPAs. These polysaccharides had no cryoprotective abilities when used alone, but resulted in enhanced viability when mixed with known permeating CPAs (such as DMSO) (Zhou et al. 1997; Almqvist et al. 2001).

Amongst the different polymers used in biotechnological and medical applications, alginates are very versatile protein-free, well characterized immuno-protecting, ionotropic matrices for immobilization of single cells (Glicklis et al. 2000) and multi-cellular aggregates (e.g. Langerhans' islets and parathyroid tissue) (Hasse et al. 1997; Schneider et al. 2005). The biocompatibility and mechanical and elastic properties of alginate micro-capsules or membranes depend on the algal source, processing methods and formulation and gelling conditions (Zimmermann et al. 2005). selecting the optimum alginate composition and cross-linking ion concentration can control cell growth and differentiation and is dictated by the application and scientific question being addressed (Simpson et al. 2004). Clinical-grade, highly purified alginate of extremely high viscosity (0.1% w/v solution in distilled water > 30 mPa) extracted by a novel process and uniformly cross-linked with Ba^{2+} has been described as an improvement for the entrapment of cells, tissues or other biologically active material (Zimmermann et al. 2005). Ultra-high viscous (UHV) alginate exhibits a uniform molecular mass distribution, does not contain short polymeric alginate chains and the high viscosity prevents the built-up of concentration gradients during alginate droplet formation and cross-linking. Cell entrapment within UHV alginate is a promising strategy for cell-based therapies and tissue engineering since it provides effective protection against the cell-mediated immune response of the host while simultaneously allowing unimpeded transfer of nutrients, oxygen, and therapeutic factors (Zimmermann et al. 2005).

2.2 NEW-GENERATION CRYOPRESERVATION SOLUTIONS

The role of the vehicle solution for the CPAs is often overlooked. It is generally assumed that conventional culture media used to nurture cells at physiological temperatures will also provide a suitable medium for exposure at low temperatures. Studies have shown that maintenance of the ionic and

hydraulic balances of cells at low temperatures can facilitate the maintenance of cell homeostasis and produce more viable cells and tissues after storage (Taylor 1982; Taylor et al. 1985). One way to achieve this balance is by careful formulation of solutions that help maintain appropriate conditions for the cells at these non-physiological temperatures (Taylor et al. 1978; Taylor et al. 1985; Baust et al. 1999; Taylor et al. 2001; Baust et al. 2002).

Traditional cryopreservation media formulations consist of a basal culture media with serum protein and DMSO supplementation. While providing for physical protection through the DMSO and protein components, the basal solutions do not provide for control/modulation of the molecular response of cells to preservation stresses. Furthermore, culture media based solutions fall short by failing to control alterations in solution pH, osmolarity, cellular generation of free radicals and energy deprivation necessary for cell survival and reduction of cellular stress during preservation.

Currently, a number of preservation solutions are available for use in the regenerative medicine field, such as UW solution (ViaSpan), Celsior, and HTK (Custodiol) (Ackemann et al. 2002; Straatsburg et al. 2002; Uhlmann et al. 2002), which were originally developed for whole organ transplantation. However, these solutions were not targeted to meet the specific, molecular requirements of individual cells and tissues. The vehicle solutions Euro-Collins and Unisol-cryoprotectant have shown to allow the cryopreservation of two vascular cell lines as monolayers, although only 50% recovery of metabolic function immediately after thawing was obtained and full recovery could only be observed 3 days post-thawing (Campbell et al. 2007).

The HypoThermosolTM line of solutions has been utilized to investigate hypothermic preservation of multiple cell types and also serves as a platform for the development of a family of cryopreservation solutions (CryoStorTM). While traditional cryopreservation media are historically exclusively “extracellular-like” with regard to ionic concentrations (high Na⁺, low K⁺), HypoThermosolTM was

designed around the concept on an “intracellular-type carrier media” as its ionic concentration was designed to balance the ions of cells during hypothermia (low Na^+ , high K^+) (Taylor et al. 1985). Studies on the cryopreservation of different cellular systems, such as hepatocytes, cord blood or stem cells, using CryoStor™ solution have reported improved cell survival and function, which have been related to a direct reduction in the level of both apoptosis and necrosis during post-thaw recovery (Baust et al. 2000; Baust et al. 2001; Baust et al. 2002; Sosef et al. 2005; Baust et al. 2006; Stylianou et al. 2006). Finally, while being a serum-free solution, the use of this type of cryopreservation media eliminates the problems related with the use of animal-derived components, such as difficulties in dealing with variability between lots of serum and the risk of transmission of potentially infectious agents to humans, which are not compatible with the development of cell-based therapies.

2.3 MINIATURISATION AND AUTOMATION

Currently, samples are typically stored in 1 to 10 ml plastic tubes or bags inside cryocontainers. Such sample volumes are disproportionately large and inconvenient for many purposes and this increases costs and restricts cryostorage capacity. Considering that the sample number will increase ten- or hundredfold with demands of emerging medical applications for cryopreserved cells and tissues, new solutions for cryostorage need to be developed.

The use of the miniaturized and divisible cryosubstrates securely attached to an electronic memory chip (micro-cryosubstrates) developed at the Fraunhofer IBMT has been proposed as a promising technical improvement of the cryopreservation process due to the possibility of miniaturisation, process automation and easy access to sample information (Zimmermann et al. 2003; Zimmermann et al. 2004; Zimmermann et al. 2005). Each IBMT’s micro-cryosubstrate is made from high-density-polyethylene and contains 30 sample

wells of 25 μ l in a plate of 43 cm. Cell suspensions, grown tissue, biopsies, encapsulated cells and 3-D aggregates can be stored in the micro-containers.

The main advantages of these systems are the avoidance of large temperature gradients and non-homogeneities, since freezing in the micro-volumes of each cryosubstrate's well takes place in a very homogeneous and highly defined way compared to the concentric temperature gradient in the cryovials (Zimmermann et al. 2004). Furthermore, the CPA can be fast and accurately added to each micro-cryosubstrate by using a pipette robot system, which results in an immediate and homogeneous distribution of the CPAs and allows to easily deal with a large number of samples.

Finally, unambiguous assignment of data records is achieved through the physical connection of a cryo-tolerant electronic memory chip to each cryosubstrate: data is stored at-sample on-chip, making these cryosubstrates valuable tools for cryobanks dealing with many samples and much information (Ihmig et al. 2006). The use of such cryosubstrates will aid the development of a secure and expandable cryobiotechnology spanning sampling for industrial-scale research cryobanks.

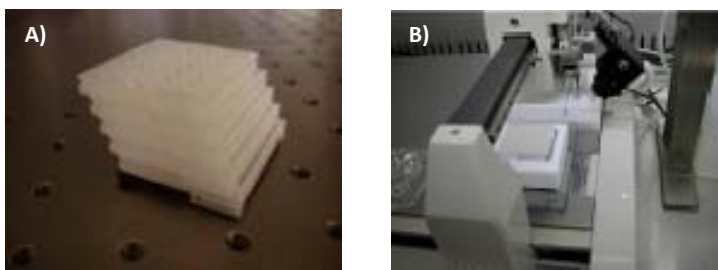


Figure 6. Micro-cryosubstrates developed at the *Fraunhofer Institut für Biomedizinische Technik IBMT*: A) set of micro-cryosubstrates; B) CPA addition to a micro-cryosubstrate using an automatic pipette robot system.

3 CRYOPRESERVATION OF HUMAN EMBRYONIC STEM CELLS (HESC)

hESC are pluripotent, immortal cells derived from blastocyst-stage human embryos (Odorico et al. 2001; Trounson 2002). These cells can be propagated indefinitely in an undifferentiated state *in vitro* and have the ability to differentiate into derivatives of all three embryonic germ layers (Thomson et al. 1998; Reubinoff et al. 2000), including neural cells, myocytes, hematopoietic cells, and insulin-secreting cells (Itskovitz-Eldor et al. 2000; Reubinoff et al. 2000; Assady et al. 2001; Kaufman et al. 2001; Kehat et al. 2001). These characteristics make hESC lines valuable for basic scientific research (Eisenberg et al. 2004; Chen et al. 2006), gene therapy (Strulovici et al. 2007), transplantation clinical therapy (Gerecht-Nir et al. 2004; Lerou et al. 2005) and pharmacological and cytotoxicity screening *in vitro* (Rohwedel et al. 2001; Gribaldo 2002).

One of the major bottlenecks for the widespread application of these cells is the lack of efficient cryopreservation protocols (Heng et al. 2006), which are required for the implementation of banks of well-characterized and safety-tested stocks of hESC. These are critical to increase patient access, reduce cost, reduce time between cell storage and use in experimental or clinical settings, permit preservation of stocks of early-passage cell lines, facilitate creation and maintenance of cellular clone banks developed from hESC lines and enhance the safety and effectiveness of these therapies (Gearhart 1998).

Efficient, reproducible, scalable and automatable methodologies that facilitate the cryopreservation of sufficient cell numbers for clinically-relevant applications must be implemented, which assure high survival, low colony fragmentation, low differentiation rates and maintenance of pluripotency post-thawing (Hunt et al. 2007; Hwang et al. 2009). Furthermore, for standardisation and quality control of cell banking (Stacey et al. 2007), cryopreservation protocols for hESC should be compatible with current good manufacturing practice (GMP) and good tissue culture practice (GTCP).

3.1 CURRENT CRYOPRESERVATION METHODS FOR hESC

As with most cells and tissues, initial studies on the cryopreservation of hESC have been empirical and based largely on methods successfully applied to other, apparently closely related cells, such as the embryo and mouse embryonic stem cells (mESC). In general, two techniques have been adopted for hESC cryopreservation, slow-rate freezing and vitrification of colonies in suspension (Reubinoff et al. 2001; Ji et al. 2004; Richards et al. 2004; Hunt et al. 2007). Both methods are plagued by poor viability and high differentiation rates upon recovery, which causes numerous problems, such as increasing the time between thawing a sample and performing experiments, clinical use or inducing selection pressures during freeze–thaw cycles due to the low survival rates (Ji et al. 2004).

Vitrification has come to be considered the preferred technique for the cryopreservation of hESC since it was first reported to yield higher survival rates when compared to slow-rate freezing by Reubinoff *et al.* in 2001 (Reubinoff et al. 2001). In 2004, Richards *et al.* have reported that vitrification of hESC clumps in closed or open pulled straws (OPS) produced significantly higher survival rates of hESC than conventional slow-rate freezing in cryovials (8%–10% vs. 75%–88%) and lower differentiation rates (Richards et al. 2004). However, vitrification protocols have several limitations preventing its widespread use for hESC (Hunt et al. 2007):

- (i) use high concentrations of a CPA that is toxic to cells at room temperature and may induce differentiation of hESC;
- (ii) require strict timing and a very small volume of hESC to get good results, which pose serious technical difficulties in developing machine automation and are clearly unsuitable for handling bulk quantities of hESC;
- (iii) are tedious to perform manually and its success depends on the researcher's level of expertise;
- (iv) introduce the possibility of contaminating hESC with infectious agents via contact with LN₂ if sealed containers are not used.

Collectively, these attributes make vitrification of hESC clumps largely unsuitable for use in preparing samples for distribution to other laboratories.

The conventional slow-rate freezing protocol of cell suspensions in cryovials using standard CPA concentrations has fewer logistic problems when compared to vitrification. However, although being efficient for the cryopreservation of mESC (Kaufman 1995), when applied to hESC this method results in low levels of recovery, low plating efficiencies, high differentiation rates and loss of pluripotency (Reubinoff et al. 2001; Kim et al. 2004; Richards et al. 2004; Heng et al. 2006), presumably due to ice crystal formation that disrupts cell-cell adhesion (Reubinoff et al. 2001; Ji et al. 2004; Richards et al. 2004).

Attempts at improving hESC cryopreservation through slow-rate freezing have focus on different strategies. In 2004, Ji *et al.* reported on improved post-thaw viability and reduction in cell differentiation for hESC frozen adherent to soluble basement membrane matrix (matrigel) or human embryonic fibroblast (hFF) feeder layers, and further improvement trehalose loading (Ji et al. 2004). In 2005, Ware et al. reported a 79% survival rate with no apparent increase in differentiation using a controlled-rate freezer and straws as containers, indicating that rapid recovery and high viability can be achieved using this controlled-rate cryopreservation technique (Ware et al. 2005). Another study reported a similar 80% survival rate and suggested that adjusting the protocol by altering the seeding temperature, cooling rate, and final temperature before plunging into LN₂ may optimize the recovery of cryopreserved hESC (Yang et al. 2005). However, because the scales of those studies were small and compatible cryovessels were still limited to certain types of straws, the techniques are difficult to apply to the large-scale cryopreservation of hESC for universal use. Moreover, in those studies, differentiation rates after thawing were either not well described or were substantially higher than those observed using vitrification. Ha *et al.* had introduced a simple procedure for the small-scale freezing of hESC using a commercial freezing container and cryovials, which has provided reasonable

survival rates (10%–30%) without using a program-controlled freezer when freezing in 5% DMSO and 10% EG in 50% fetal bovine serum (FBS) (Ha et al. 2005). However, some concerns remained for clinical applications when serum is used as previously described. Also, survival and differentiation rates of frozen/thawed hESC were unstable and variable depending on the level of researcher's expertise since hESC clumps easily dispersed into pieces during equilibration and freezing/thawing. Lee *et al.* have focused on a synthetic serum substitute for replacing animal serum and stepwise equilibration for reducing mechanical and osmotic damages. The slow-rate freezing method proposed using stepwise equilibration of serum replacer was able to cryopreserve a large amount of hESC at once by enzymatic dissociation and use of cryovials (Lee et al. 2008). Other strategies have focused on the addition to the cryopreservation medium of sugars such as trehalose (Wu et al. 2005) or ECM proteins such as collagen and laminin (Kim et al. 2004), or more recently the presence of the caspase inhibitor Z-VAD-FMK (Heng et al. 2007) or the Rho-associated kinase ROCK inhibitor (Martin-Ibanez et al. 2008). Such strategies have been shown to produce modest improvements in the recovery of colony fragments and reduce the levels of cellular differentiation.

A summary of studies involving the cryopreservation of hESC is presented in Table III. Although some improvements have been reported, efficient, standardised protocols compatible with new stem-cell-based therapies are still lacking.

Table III. Summary of studies involving the cryopreservation of hESCs.

Cell state	Reference	Cryodevice, Cryomedium and Freezing Method	Main Results
Colony clumps	(Reubinoff et al. 2001)	OPS Vitrification (250 µl straws): - Subsequent incubation in 2 vitrification solution containing increased concentrations of DMSO, EG and sucrose; - Submersion in LN ₂ .	- Clumps recovered upon thawing and gave rise to hESC colonies after plating; - Significantly smaller colonies with increased level of differentiation compared with control colonies.
	(Kim et al. 2004)	Slow-rate freezing (250 µl straws): - In 90% FBS + 10% DMSO; - Cooling at 1°C/min to -80°C (seeding at -7°C).	- Significantly increased survival (~37%) of hESCs by addition of type IV collagen to the freezing medium compared to control and laminin-treated groups (~22% survival); - Significantly lower rates of differentiation by addition of collagen or laminin.
	(Richards et al. 2004)	Vitrification: 8 different protocols (based on (Reubinoff et al. 2001)) in a combination of: - Close straws versus open straws; - FCS versus HAS; - Liquid N ₂ versus vapour N ₂ .	- High survival (~80%), low differentiation and maintenance of pluripotency; - Manual manipulation of individual colonies during passaging difficulties the generation of sufficient numbers of cells for clinical application.
	(Ha et al. 2005)	Slow-rate freezing/ rapid warming (2 ml cryovials): - In FBS + 5%, 50% or 95% DMSO, EG or glycerol; - Cooling -70°C and storage in LN ₂ .	The optimal combination obtained with 5% DMSO + 50% FBS + 10% EG (~30% survival).
	(Ware et al. 2005)	Slow-rate freezing / rapid warming (250 µl straws): - In culture medium (25% FBS) + 10% DMSO; - Straws held at RT for 20 min, held for 2-5 min at seeding temperature and touched with LN ₂ forceps, frozen to -30°C and plunged into LN ₂ .	- Highest rates of survival (~80%) achieved with a commercial freezing device; - Critical factors are ice seed (at -7°C to -10°C), cooling rate between 0.3 and 1.8°C/min and rapid warming rate (RT water).
	(Yang et al. 2005)	Slow-rate freezing / rapid warming (2ml cryovials): - In 60% culture medium + 30% FBS + 10% DMSO; - Cooling at different rates (0.3, 0.5 or 2°C/min), to different terminative temperatures before plunging into LN ₂ (-35, -60 or -80°C), with (at -10°C) or without seeding.	- Highest survival rate (81.8%) obtained when the samples are cooled at 0.5°C/min to -35°C (seeding at -10°C) and then plunged into LN ₂ ; - The cryopreserved hESCs retained the properties of pluripotent cells.
	(Wu et al. 2005)	Slow-rate freezing / rapid warming (cryovials): - In 90% knock out serum replacement + 10% DMSO; - Cryomedium supplement with 0.2g/L trehalose; - Cooling at 1°C/min to -80°C.	- Improve in the recovery rate from 15% to 48% by trehalose addition to the cryomedium; - The cryopreserved hESCs retained the properties of pluripotent cells.

	(Li et al. 2008)	<p>Bulk Vitrification (in cell strainers):</p> <ul style="list-style-type: none"> - Clumps (diameter > 70µm) in cell strainer are transferred between vitrification solutions (according to (Reubinoff et al. 2001)) and finally to LN₂ directly. 	<ul style="list-style-type: none"> - Bulk-vitrified hESCs exhibited high survival rate (94.3%) comparable with the OPS vitrification method; - The Bulk-vitrified hESCs retained the properties of pluripotent cells.
	(Lee et al. 2008)	<p>Slow-rate freezing / rapid warming (cryovials):</p> <ul style="list-style-type: none"> - In 90% knock out serum replacement + 10% DMSO; - One-step versus stepwise equilibration method; - Cooling in cryo-container versus programmable-controlled freezer (at 1°C/min to -80°C). 	<ul style="list-style-type: none"> - Highest survival rate achieved with stepwise equilibration and programmable-controlled freezer; - a commercial freezing device; - The cryopreserved hESCs retained the properties of pluripotent cells, the karyotypes and expression of three germ layer markers.
	(Ji et al. 2004)	<p>Slow-rate freezing / rapid warming (cells adherent to well-plates):</p> <ul style="list-style-type: none"> - Adherent cells on matrigel or MEFs versus freely-suspended clumps; - Cooling at 1°C/min to -80°C in serum and DMSO and then transfer to the vapour-phase of LN₂; - Cells loaded with 35mM trehalose 2 days prior to freezing. 	<ul style="list-style-type: none"> - Cell viability increased by over an order of magnitude and reduced differentiation; trehalose further improved cell viability; - The recovery of adherent hESCs frozen on microcarriers improved by ~10 times when compared to hESCs frozen as free clumps;
Adherent cells	(Heng et al. 2007)	<p>Slow -rate freezing / rapid warming (cells adherent to well-plates):</p> <ul style="list-style-type: none"> - In culture medium (20% FBS) + 10% DMSO with or without 100mM of the caspase inhibitor Z-VAD-FMK; - Cooling to -80°C in an insulated styrofoam box. 	<ul style="list-style-type: none"> - Addition of 100mM Z-VAD-FMK increased the survival rate from 9.9% to 18.7%; - Addition of 100mM Z-VAD-FMK had no effect on the spontaneous differentiation of cryopreserved hESCs after thawing.
	(Nie et al. 2009)	<p>Slow -rate freezing / rapid warming (cells adherent to microcarriers in 1ml cryovials):</p> <ul style="list-style-type: none"> - Colonies adherent to microcarriers versus freely-suspended clumps; - In 60% culture medium + 30% FBS + 10% DMSO; - Cooling to -80°C in commercial freezing container and stored in LN₂. 	<ul style="list-style-type: none"> - The recovery of adherent hESCs frozen on microcarriers improved by ~1.7 times when compared to hESCs frozen as free clumps; - hESCs remained undifferentiated after cryopreservation on microcarriers.
Single-cells	(Martin-Ibanez et al. 2008)	<p>Slow-rate freezing / rapid warming (1 ml cryovials):</p> <ul style="list-style-type: none"> - In 90% culture medium (without serum) + 10% DMSO; - Cryo- / post-thawing medium supplement with 10µM ROCK inhibitor Y-27632; - Cooling at 1°C/min to -80°C and stored in N₂; 	<ul style="list-style-type: none"> - Significant increase in hESCs survival rate and efficiency of colony formation through addition of 10µM ROCK inhibitor Y-27632; - The cryopreserved hESCs retained the properties of pluripotent cells and the potential to differentiate into derivatives of all three germ layers after embryoid bodies formation.

4 SCOPE OF THE THESIS

The work presented in this thesis focused on the development of effective animal-component-free, GMP-compliant, scalable cryopreservation methodologies for complex cell systems, which can generate sufficient numbers of well-characterized samples for research, therapeutic and/or pharmacological applications. Specifically, cryopreservation strategies for functional 2-D monolayers and 3-D aggregates were addressed. The final aim was to obtain improved cryopreservation protocols for hESC, ensuring high survival, low colony fragmentation, low differentiation rates and maintenance of pluripotency post-thawing.

To achieve this, an integrated approach was developed by addressing specific steps within the general cryopreservation workflow, namely the pre-freezing conditions, freezing method and post-thaw recovery conditions. As model systems for studying different combined strategies for cryopreservation of 2-D monolayers and 3-D aggregates, we used:

(i) mouse neuroblastoma N2a and human colon adenocarcinoma Caco-2 cell lines, due to their capacity to differentiate into well characterized cell types and their worldwide recognized usefulness as in-vitro tools for drug screening assays where the availability of “ready-to-use” cells is a critical issue;

(ii) primary cultures of brain neurospheres due to their well-organized cellular spatial arrangement, constituted by different cell types and interactions, with patterns of morphological differentiation resembling the ones present in intact brain tissue and because they are suitable for growth and maintenance under well-defined and reproducible conditions, namely, in bioreactors.

Since maintenance of colony integrity has shown to be a crucial requirement for the successful cryopreservation of hESC (Ji et al. 2004), the effect of cell entrapment within highly purified, ultra-high viscous (UHV) alginate

(Zimmermann et al. 2005) was evaluated as the main strategy for avoiding the commonly observed colony fragmentation and loss of cell-cell/matrix interactions post-thawing.

Traditional cryopreservation media formulations consist of a basal culture media supplemented with serum protein, which is not compatible with development of a GMP-compliant cryopreservation protocol. Thus, standard cell culture media was compared with protein- and serum-free CryoStorTM solution as the vehicle solution for the CPA.

Process automation was addressed by testing the use of miniaturized and divisible cryosubstrates developed at the Fraunhofer IBMT together with high-speed dispensing pipette robots.

A summary of the bottlenecks in the steps of the cryopreservation workflow and the cell systems that will be focused in this thesis as well as the strategies that will be employed to address them are summarized on tables IV and V.

Development of integrated strategies for the efficient culture and cryopreservation of the complex cell systems addressed in this thesis will support the implementation of high-throughput cell-based assays and routine cryopreservation practices during preparation of engineered cells and tissues and facilitate the establishment of tissue banks for tissue engineering and clinical applications.

Table IV. Summary of the main bottlenecks in the steps of the cryopreservation workflow that will be addressed in this thesis and the strategies applied to overcome them.

Workflow Step		Bottlenecks	Strategies
Pre-freezing Conditions	Cryomedium	<ul style="list-style-type: none"> - Potential hazard of transmission of infectious agents through animal derived products; - Compromised reproducibility due to the use of different batches of non-defined reagents. 	Cryopreservation in serum-free cryomedium (CryoStor TM , VitroHES).
	CPA	DMSO cytotoxicity at different concentrations depending on the cell type.	<ul style="list-style-type: none"> - Reduction of DMSO concentration; - Replacement by non-toxic CPAs (e.g. glycerol).
	Cryovessel	Traditional storage volumes (1 ml cryovials): <ul style="list-style-type: none"> - higher susceptibility to heterogeneous ice nucleation; - not ideal for high-throughput cell banking; 	Reduction of cryovessel volume through the use of micro-cryosubstrates (25 µl/well).
Freezing Method	Slow-rate Freezing	Standard protocols are inefficient for complex cell-systems (e.g. 2-D monolayers or 3-D aggregates).	<ul style="list-style-type: none"> - Cell entrapment within UHV alginate; - Cryomedium containing anti-apoptotic factors ; - Cryopreservation in micro-cryosubstrates.
	Vitrification	<ul style="list-style-type: none"> - Labor-intensive and tedious to perform manually; - Cryostraws are unsuitable for handling bulk quantities and developing process automation; - Potential hazard of transmission of infective agents into cells. 	<ul style="list-style-type: none"> - Surface-based vitrification; - Use of closed cryovessels.
Post-thaw Recovery	Assessment of Recovery	<ul style="list-style-type: none"> - Occurrence of cell death within hours to days post-thawing; - Absence of effective, consistent and validated protocols for assessment of post-thaw recovery. 	<ul style="list-style-type: none"> - Cryomedium containing anti-apoptotic factors (CryoStorTM); - Addition of apoptosis inhibitors to the cryo- and post-thawing medium; - Evaluation of proper time points for evaluation of cell recovery; - Assessment of cell recovery with proper, cell-specific indicators.

Table V. Summary of the main bottlenecks related to the cell types that will be addressed in this thesis and the strategies applied to overcome them.

Cell		Bottlenecks	Strategies
Type	Primary Cultures	<ul style="list-style-type: none"> - Loss of cell viability and metabolic activity; - Loss of cell-cell / cell-matrix junctions; - Loss of functionality and/or metabolic interactions. 	<ul style="list-style-type: none"> - Cryopreservation of 3-D aggregates mimicking the tissue of origin; - Entrapment within micro-capsules of UHV alginate; - Cryomedium containing anti-apoptotic factors (CryoStorTM).
	hESC	<ul style="list-style-type: none"> - Loss of viability; - Uncontrolled differentiation; - Colony disintegration; - Loss of pluripotency; - Loss of capacity to differentiate into derivatives of all three germ layers. 	<ul style="list-style-type: none"> - Slow-rate freezing of colonies adherent to well-plates; - Vitrification of adherent colonies; - Slow-rate freezing of 3-D clumps; - Cell entrapment within UHV alginate; - Cryomedium containing anti-apoptotic factors (CryoStorTM); - Addition of apoptosis inhibitors to the cryo- and post-thawing medium.
Culture Strategy	2-D Monolayers	<ul style="list-style-type: none"> - Loss of membrane integrity and cell detachment immediately after thawing; - Breakage of cell-cell and cell-matrix interactions; - Drop in viability within 24 hrs post-thawing. 	<ul style="list-style-type: none"> - Entrapment beneath a layer of UHV alginate; - Cryomedium containing anti-apoptotic factors (CryoStorTM).
	3-D Aggregates	<ul style="list-style-type: none"> - Loss of cell-cell / cell-matrix junctions and aggregates disintegration; - Loss of functionality and cell-cell specific interactions; - Loss of membrane damage and metabolic activity. 	<ul style="list-style-type: none"> - Entrapment beneath a layer of UHV alginate; - Cryopreservation medium containing anti-apoptotic factors (CryoStorTM).

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CRYOPRESERVATION OF SINGLE-CELL SUSPENSIONS

**Cryopreservation in Micro-volumes: Impact upon Caco-2 Colon
Adenocarcinoma Cells Proliferation and Differentiation**

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ABSTRACT

The recent advances on cell-based therapies require new approaches for cell cryopreservation that can deal with large number of samples and provide specific conditions for each cell type. The miniaturisation of the sample volume from the typically used 1 millilitre down to 25 micro-litres using 30 wells micro-cryosubstrates represents a promising improvement for the freezing process due to the possibility of process automation, data handling and physical access to each well without thawing the whole cryosubstrate. This novel system was evaluated for the storage of Caco-2 colon adenocarcinoma cells, which differentiate spontaneously after long-time in culture. The impact of the cryovessel small volume upon post-thawing membrane integrity of the cells and their capacity to proliferate and differentiate was studied. Two different cryoprotectants (CPAs) commonly employed, dimethyl sulfoxie (DMSO) and glycerol, were evaluated as well as the possibility of decreasing their concentration from the 10% concentration, usually used, down to 3% (v/v). The process automation by pipette robotic addition of the CPA to the micro-cryosubstrates was also evaluated. The micro-cryosubstrates have proven to be at least as efficient as typical 1 ml cryovials for cryopreservation of Caco-2 cells using either DMSO or glycerol. Compared to the manual process, the automatic addition of glycerol to the micro-cryosubstrates allowed higher cell viabilities after thawing while with DMSO no significant changes were observed. DMSO has shown to be more effective than glycerol in maintaining high post-thaw cell membrane integrity, either in micro-cryosubstrates or cryovials, for any of the concentrations tested. The ability of DMSO in maintaining high cell membrane integrity post-thawing was confirmed by long-time (up to 22 days) proliferation and differentiation studies performed with cells cultured immediately after thawing.

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1. INTRODUCTION

The interest in multipotent stem cells from embryonic and adult sources has led to the increasing importance of cell-based therapies, namely regenerative and reparative medicine (Keller et al. 1999; Glicklis et al. 2000; Gerlach et al. 2002; Daley et al. 2003; Henningson et al. 2003; Schneider et al. 2005). For such biomedical applications, efficient preservation technologies are required in order to assure maximum cell recovery and maintenance of cell function and differentiation potential after long-time storage, and facilitate storage of biomedically relevant cell lines for the establishment of cell banks (Gearhart 1998).

Cryopreservation in liquid nitrogen at temperatures ranging from -130°C to -196°C is the currently used technique to preserve living cells and tissues for long periods of time (Grout et al. 1990; Özkavukcu et al. 2002; Woods et al. 2004). The standard protocol for cryopreservation of mammalian cell lines consists in freezing the cells in 1 milliliter cryovials (made from polypropylene) in a mixture of growth medium, fetal bovine serum (FBS) (concentrations ranging from 5% to 20% (v/v)), and DMSO usually at 10% (v/v) concentration (McLellan et al. 1995). Slow-freezing and rapid-thawing rates are regularly applied to minimize intracellular ice formation (IIF) and osmotic damage (Freshney 1994). While suitable for the routine storage of the majority of the cell lines currently used on research, this protocol must be optimized when dealing with cells intended to be used on cell-based treatments (Reubinoff et al. 2001; Ha et al. 2005). During the freezing process, cellular changes and molecular alterations may occur as a result of the osmotic stress, stresses on cell junction and cell-transport systems, which can contribute to a loss of cell viability and affect cell function or its differentiation potential (Frederickx et al. 2004; Kim et al. 2004; Woods et al. 2004). Thus, an efficient cryopreservation process must assure not only high cell viability post-thawing, but also a short time lag between thawing and full cell proliferation and

metabolic activity. In the case of cells which possess the capacity to differentiate, e.g. stem cells, the additional capability of efficiently starting differentiation must be also preserved during cryopreservation.

Several reports in the literature describe different alternative cryopreservation technologies that have been tested for different types of cells intended to be used on specific applications, such as freezing cells adherent to structured glass and silicon substrates (Hornung et al. 1996) or entrapment on matrices (Ji et al. 2004) or gels beads (Hubel et al. 1991; Mahler et al. 2003).

The use of the micro-cryosubstrates developed at the Fraunhofer IBMT has also been proposed as a promising technical improvement of the cryopreservation process due to the biophysical and logistical advantages of miniaturisation, process automation and easy access to fractions of the sample, allowing for the manipulation of large number of samples (Zimmermann et al. 2003; Zimmermann et al. 2004; Zimmermann et al. 2005). Micro-cryosubstrates are made from high-density-polyethylene using injection-molding and contain 30 sample wells of 25 μ l net volume in a plate of 4×3 cm. The main advantages of these systems are the avoidance of large temperature gradients and non-homogeneities, since freezing in the micro-volumes of each cryosubstrate's well takes place in a very homogeneous and highly defined way compared to the concentric temperature gradient in the cryovials (Zimmermann et al. 2004). Furthermore, the CPA can be fast and exactly added to each micro-cryosubstrate by using a pipette robot system, which results in an immediate and homogeneous distribution of the CPAs and allows to easily deal with a large number of samples. Finally, it is possible to physically attach a cryo-tolerant memory chip to each cryosubstrate so that each sample's data is stored at-sample on-chip, making these cryosubstrates valuable tools to be used in cryobanks where there is the need to deal with a great number of samples and sample information (Ihmig et al. 2006).

In this study we investigated among others the effect of reduced sample's volume (comparison of 1 ml and 25 μ l net volume) to the cryopreservation of Caco-2 cells in suspension. This cell line was used as a model due to its capacity to spontaneously differentiate upon confluence to a cell type exhibiting characteristics typical of small intestinal epithelial cells, which can be easily assessed, both morphologically and functionally (Delie et al. 1997). We also investigated the replacement of DMSO by a less toxic CPA, glycerol, and the effect of reducing the CPA concentration. Finally, the automatic addition of the CPA to the micro-cryosubstrates by using a pipette robot system as an alternative to manual filling was also studied. All the parameters were evaluated for their effect upon cell membrane integrity post-thawing as well as on cell proliferation and differentiation during long-time (22 days) culture immediately after thawing.

2. MATERIALS AND METHODS

2.1. MATERIALS

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and trypsin-EDTA (1x solution) were obtained from GIBCO (Glasgow, UK). Culture flasks, Thermanox plastic coverslips and 1 ml cryovials were obtained from Nunc (Roskilde, Denmark). 96 and 4 well-plates were obtained from Sarstedt (Newton, USA). DMSO, glycerol, the fluorescent dyes and 4-methylumbelliferyl phosphate (4-MUP) were obtained from Sigma (Steinheim, Germany). The BrdU-ELISA Kit was obtained from Roche (Mannheim, Germany). The CyQuant Assay Kit was obtained from Molecular Probes (Eugene, USA). Other chemicals were of the purest grade available from regular commercial sources.

2.2. CELL CULTURE

Caco-2 cell line was obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen (Barunshweig, Germany). The cells were maintained in DMEM supplemented with 10% (v/v) heat inactivated FBS and 4.5

g/l glucose at 37°C in a humidified atmosphere of 5% CO₂ in air. All the cells used in this study were obtained from passages 12 to 30.

2.3. CELL CRYOPRESERVATION

Upon confluence, cells were detached from the culture flasks using trypsin/EDTA and resuspended in culture medium. Cell suspension was centrifuged (200 *g*, 5 min at 4°C) and total cell number was determined by the trypan blue exclusion method using a 0.1 % (v/v) solution prepared in PBS and counting cells in a Fuchs-Rosenthal haemocytometer (Brand, Wertheim, Germany). The cells were then re-suspended in the culture medium volume necessary to have a cell concentration of 1×10^6 cells/ml. Cell membrane integrity before freezing was always higher than 92% as assessed through a fluorescent membrane integrity test described below. The micro-cryosubstrates and cryovials were manually filled with the cell suspension. The CPAs, DMSO or glycerol (40% v/v), in the concentrations to be tested (10%, 5% and 3% (v/v)), were either mixed with the cell suspension prior to the filling of the micro-cryosubstrates and cryovials, or automatically added to the micro-cryosubstrates using the micro-pipetting system Nano-Plotter NP1.2 (GeSiM mbH, Germany) (as described in previous work by (Zimmermann et al. 2003; Zimmermann et al. 2004)) after filling with the cell suspension to achieve the final concentration required in each experiment. The micro-cryosubstrates and cryovials were placed at 4°C during 20 minutes for CPA equilibration in medium, frozen to –80°C in an isopropanol-based freezing system, “Mr. Frosty”, Nalgene (NY, USA), at a rate of cooling very close to 1°C per minute (accordingly to the manufacture’s specifications), and stored in the gas phase of a liquid Nitrogen storage reservoir for 30 days.

2.4. POST-THAW STUDIES

The micro-cryosubstrates and cryovials were quickly thawed by placing them in a CO₂ incubator (37°C, 5% CO₂ in air) or a water bath (37°C), respectively. The thawed cells were either (i) used for a membrane integrity assay immediately after thawing or (ii) inoculated in 96 or 4 well-plates for proliferation and differentiation studies. The proliferation assays and the measurement of alkaline phosphatase activity and cell number were performed both for cells cryopreserved in micro-cryosubstrates filled manually and for cryovials with 10% or 5% DMSO or 10% glycerol. Cells which had not been cryopreserved before the inoculation in micro-plates were used in each assay as a control. The cells were diluted in culture medium to a final concentration of 5×10^3 cells/cm², inoculated in sterile 96 well-plates and cultured for 22 days. The number of thawed cells and respective control was determined using a fluorescence test in order to obtain the same cell concentration in all samples on the micro-plates at inoculation. At determined time points, the micro-plates were used to assess proliferation, alkaline phosphatase activity and cell number. Cell growth in micro-plates was monitored during the 22 days of culture by visualization on an inverted microscope (Leica DM IRB). To evaluate differentiation scanning electron microscopy (SEM) was also performed. For those studies, 1×10^5 cells were seeded on Thermanox coverslips in 4 well-plates and cultured for 17 days. The culture medium in the 96 and 4 well plates was changed every 4 days during cell culture.

2.4.1. Cell Membrane Integrity Assay

The quantitative assessment of the cell plasma membrane integrity immediately after thawing was done using the enzyme substrate fluorescein diacetate (FDA) and the DNA-dye ethidium bromide (EB) as described in the literature (Dankberg et al. 1976). This assay is based on the fact that only cells with intact plasma membrane can accumulate fluorescein, which is enzymatically split from the nonfluorescent substrate FDA, rendering the cells a green

fluorescence; conversely, cells with damaged membranes become permeable to EB whose nuclei it stains with a red fluorescence. By combination of these fluorescent dyes, one can distinguish microscopically between cells with compromised membranes, red cells, and cells with undamaged membranes, which fluoresce green. The fluorescent solution was prepared by diluting FDA and EB in DMEM to a final concentration of 19.2 μM and 25 μM , respectively. From each cryosubstrate and cryovial, the cell suspension was diluted 1:4 in the fluorescent solution and the number of green and red cells determined by haemocytometer cell counting on an inverted microscope (Leica DM IRB, Wetzlar, Germany) under fluorescence light (blue excitation range: 450-490nm).

2.4.2. Proliferation Assay

The proliferation capacity of cells cultured for 22 days in 96 well-plates after cryopreservation was evaluated through a BrdU-ELISA colorimetric immunoassay (from Roche, Mannheim, Germany). The incorporation of the pyrimidine analogue 5-bromo-2'-deoxyuridine (BrdU) in the place of thymidine into the newly synthesized DNA of proliferating cells was measured according to the manufacturer's instructions. The reaction product was quantified by measuring the absorbance at 450 nm using a scanning multi-well spectrophotometer (Spectramax 340, Beckman Coulter, USA). The absorbance values measured directly correlate (in the range of 1×10^3 to 1×10^5 cells/well) to the amount of DNA synthesis and thereby to the number of proliferating cells in the respective well-plates.

2.4.3. Differentiation Assays

The differentiation of the cells during long-time culture in 96 and 4 well-plates after cryopreservation was assessed by measurement of the activity of the brush-border enzyme alkaline phosphatase or through electron microscopic examination of the cell morphology.

2.4.3.1. Measurement of Alkaline Phosphatase Activity.

The activity of the brush-border enzyme alkaline phosphatase on cells cultured in 96 well-plates was measured by the reaction with the substrate 4-MUP, which becomes fluorescent when the phosphate group is removed by the hydrolysis of the enzyme (as described in the literature (Watanabe et al. 1979) with some modifications). Briefly, the supernatant was carefully sucked off and cells were incubated for 1.5 hours at 37°C (protected from light) with 100 µl of equal volumes of 1 mM 4-MUP in 20 mM HEPES/NaOH pH 9.0 and 20 mM HEPES/NaOH pH 9.0. After transferring 75 µl of the supernatant into a 96 well microplate, the enzyme reaction was detected by measuring the fluorescence at 360 nm excitation and 535 nm emission in a fluorescence plate reader (Cary Eclipse Fluorescence Spectrofluorimeter, Varian, USA). Substrate without cells was used as a blank and a calibration curve was obtained using bovine alkaline phosphatase at concentrations ranging from 0.5 to 20 µg/ml. The enzyme activity is expressed as milliunits of enzyme per cell. One unit is defined as the activity that hydrolyzes 1 µmole of substrate per minute under the experimental conditions.

2.4.3.2. Cell Number Quantification

The number of cells expressing the brush-border enzyme alkaline phosphatase was quantified by the CyQuant assay kit (from Molecular Probes, Eugene, USA) according to the manufacturer's instructions. This assay is based on the green fluorescence exhibited by the CyQuant GR dye when bound to cellular nucleic acids, which was measured at 480 nm excitation and 520 nm emission in a fluorescence plate reader. A calibration curve was obtained by generating a dilution series of cells in the wells of a 96 well-plate at concentrations ranging from 2.5×10^2 – 2.5×10^5 cells/ml. The cell density was determined by haemocytometer cell counting using trypan blue exclusion dye.

2.4.3.3. Electron Microscopy

After culturing for 17 days in Thermanox coverslips the cells were prepared for SEM as described elsewhere (Katsen et al. 1998). Briefly, this procedure includes washing in HEPES buffer, fixation in glutaraldehyde, post-fixation in osmium tetroxide, tannic acid and uranyl acetate, dehydration in ethanol, critical point drying and sputtering with metal. After SEM examinations in the secondary electron mode, the same preparations were submitted to a further treatment for block-face-SEM (Denk et al. 2004). The cells were embedded in epoxy resin, cross-sectioned with ultra-microtome and then coated with carbon and studied by SEM in backscattered electron mode. SEM and block-face-SEM studies were performed on an field emission scanning electron microscope type FESEM XL30 (Phillips, USA).

2.4.4. Data Presentation/Analysis

Each experimental condition represents $n \geq 6$ experimental replicates (minimum of 2 experimental repeats with 3 sample replicates per experiment). Error bars denote the standard deviation of the mean. Comparison of mean values between groups was performed using analysis of variance (ANOVA), with $P < 0.05$ considered statistically significant. Representative photographs are depicted.

3. RESULTS

3.1. EFFECT OF CRYOPRESERVATION ON THE CELL MEMBRANE INTEGRITY

Three different approaches for cell freezing were tested concerning their effect on the post-thaw membrane integrity of the cells: manually filled and automatically filled micro-cryosubstrates as well as 1 ml cryovials.

Figure 1 shows the effect of the cryopreservation system on the viability of cells which have been frozen with either DMSO or glycerol as the CPA.

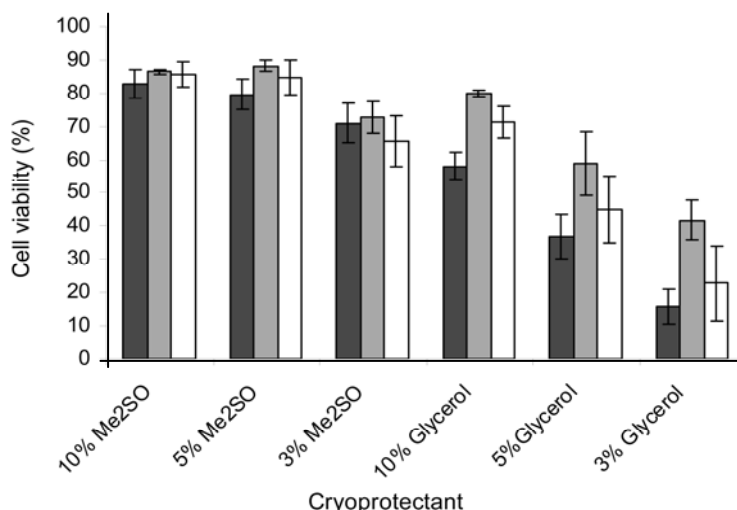


Figure 1. Effect of the sample volume on cell viability of Caco-2 cells when cryopreserved with DMSO or glycerol at different concentrations in manually (black bars) or automatically (grey bars) filled micro-cryosubstrates or cryovials (open bars).

Cells with post-thaw damaged plasma membrane were assumed to be non-viable while cells with intact membranes were assumed to be viable. Thus, the term “cell viability”, which reflects/refers to cell membrane integrity, will be used from here on and represents the number of cells with intact membranes as a percentage of the total number of cells recovered after thawing. The calculations for the percentage of cell recovery (total number of cells recovered as a percentage of cells frozen) were also done and always higher than 95% for all samples examined.

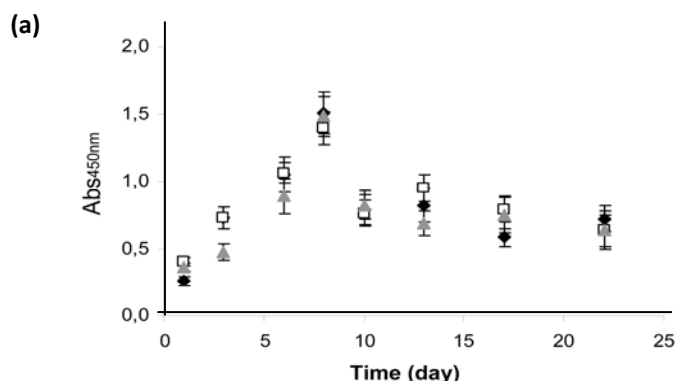
No significant difference was found on the percentage of viable cells recovered after cryopreservation in either micro-cryosubstrates or in cryovials, when DMSO or glycerol were used as the CPA. Nevertheless, DMSO has shown to be more effective than glycerol in minimizing freezing injury for any of the concentrations tested: approximately 85% cell viability was obtained when freezing with either 10% or 5% DMSO whereas a decrease from 5% to 3% led to a lost in cell viability of approximately 10%. When 10% glycerol was used as the

CPA, cell viability after thawing was marginally lower than the values obtained with DMSO for automatically filled micro-cryosubstrates while freezing in manually filled micro-cryosubstrates or in cryovials lead to lower cell viabilities. As the concentration of glycerol was further decreased from 10% to 5% and 3%, cell viability decreased considerably and the values obtained were much lower than those obtained with equivalent concentrations of DMSO, irrespective of the filling method or vessel: cell viability dropped approximately 20% when the concentration of glycerol decreased from 10% to 5% and further from 5% to 3%.

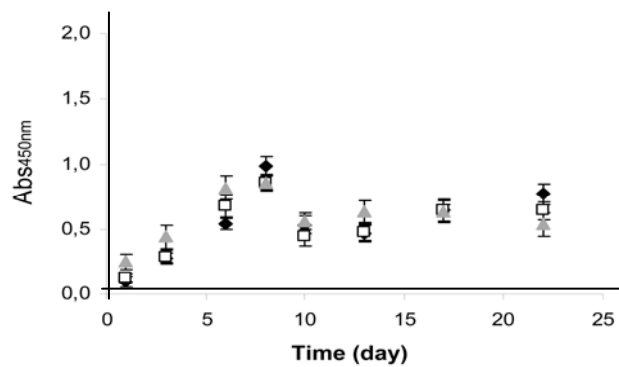
Concerning the automation of the filling process for the micro-cryosubstrates, no difference was found on the viability of cells frozen in manually or automatically filled micro-cryosubstrates when DMSO was used as the CPA. On the other hand, when glycerol was used, viability values obtained for cells frozen in automatically filled micro-cryosubstrates were approximately 20% higher than those obtained for cells frozen in manually filled micro-cryosubstrates.

3.2. EFFECT OF CRYOPRESERVATION UPON CELL PROLIFERATION CAPACITY

Figure 2 illustrates the proliferation capacity of Caco-2 during long-term post-thaw culture.



(b)



(c)

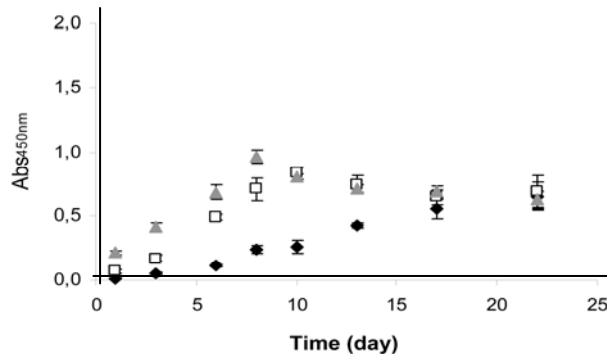


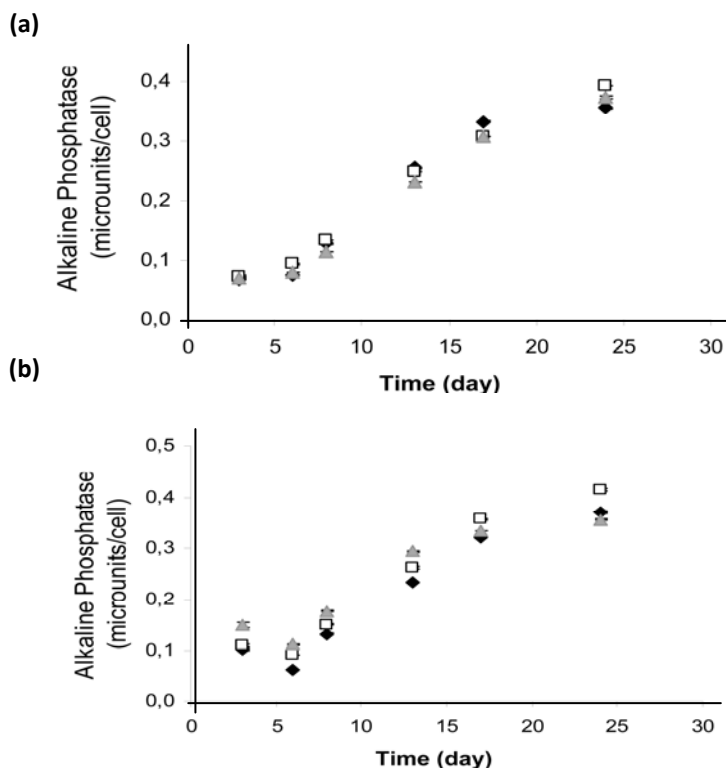
Figure 2. Proliferation of Caco-2 cells during 22 days of culture, after thawing. Cells were cryopreserved in micro-cryosubstrates (black diamonds) or cryovials (white squares) with 10% DMSO (a), 5% DMSO (b) or 10% glycerol (c) and, after thawing, immediately inoculated in 96 well-plates for proliferation assays. As controls (grey triangles) cells which had not been cryopreserved before inoculation were cultured for long-time.

As shown in Figure 2, for 10% or 5% DMSO, no significant difference was found on the proliferation capacity of cells that had been cryopreserved in micro-cryosubstrates or cryovials and control cells: the number of proliferating cells in culture increased after 1 day of inoculation and a maximum was obtained on the 8th day in culture (Figures 2a and 2b), corresponding to cell confluence as observed by examining the cultures on an inverted microscope (data not shown). The number of proliferating cells in culture decreased after confluence had been reached; however, the cells continued to proliferate and, after 22 days in culture, multilayers were formed, as assessed by microscopic examination.

For cells that had been cryopreserved with 10% glycerol in cryovials the proliferation profile was similar to the one obtained for control cells, but the absorbance values measured were lower indicating a lower number of proliferating cells in culture until confluence was reached (Figure 2c); furthermore, it is worthwhile to mention that there was a delay on the achievement of the maximum cell number for glycerol cryopreserved cells in cryovials. For cells which had been cryopreserved in micro-cryosubstrates, the number of proliferating cells in culture was always lower than those found for control cells and cells cryopreserved in cryovials and an even higher delay on the attainment of the maximum cell number was observed.

3.3. EFFECT OF CRYOPRESERVATION UPON CELL DIFFERENTIATION CAPACITY

The functional differentiation of the cells during long-time culture after thawing was assessed by measuring the activity of the brush border enzyme alkaline phosphatase.



(c)

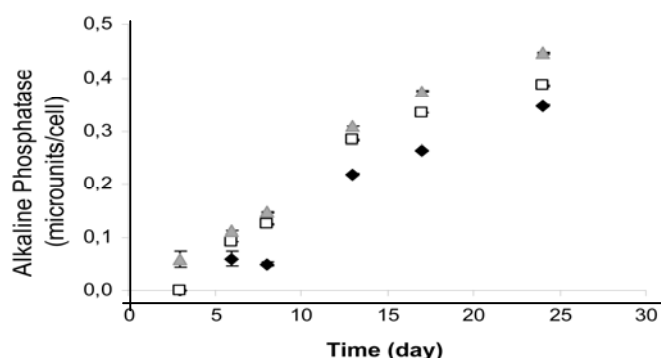


Figure 3. Differentiation of thawed Caco-2 cells assessed by measurement of the activity of alkaline phosphatase during 22 days of culture. Cells were cryopreserved in micro-cryosubstrates (black diamonds) or cryovials (white squares) with 10% DMSO (a), 5% DMSO (b) or 10% glycerol (c) and, after thawing, immediately inoculated in 96 well-plates for proliferation assays. As controls (grey triangles) cells which had not been cryopreserved before inoculation were cultured for long-time.

As observed in Figure 3, cells which had been cryopreserved in micro-cryosubstrates or in cryovials with either 10 or 5% DMSO, show an alkaline phosphatase activity during growth similar to control cells: the enzyme activity was low during the phase of active growth and started to increase after confluence had been reached (8th day of culture).

For glycerol at 10% concentration, a similar activity profile was observed; however, the activity values obtained for micro-cryosubstrates and, to a lesser extent, so for cryovials, was always lower when compared with DMSO cryopreserved cells.

The morphology of the Caco-2 cells after 17 days of culture was investigated by SEM in secondary electron mode and block-face-SEM in backscattered electron mode (Figures 4 and 5).

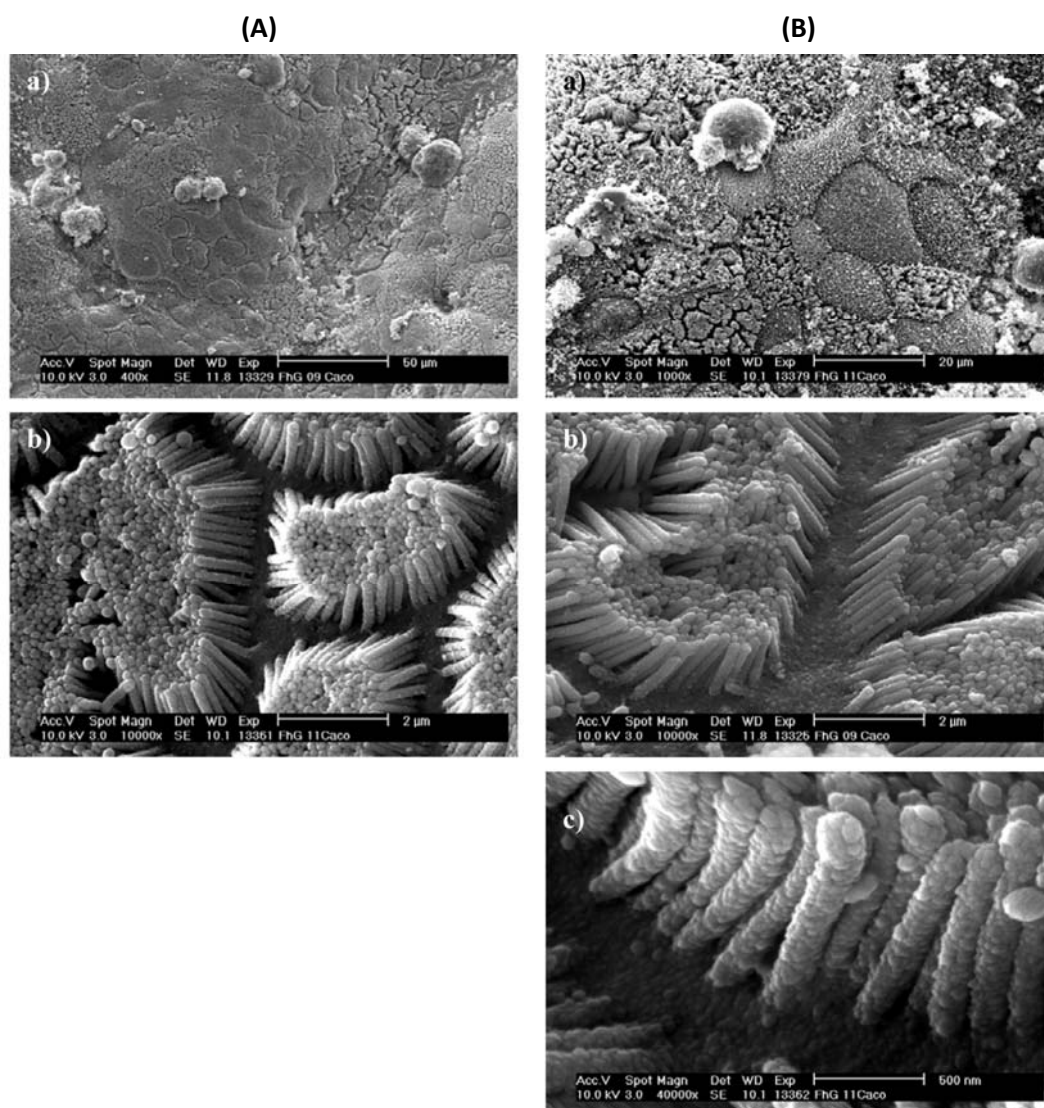


Figure 4. SEM of monolayers of Caco-2 cells after 17 days in culture. The typical features of differentiation in Caco-2 cells are shown for both control cells (A) and cells which had been cryopreserved in micro-cryosubstrates with 5% DMSO before culture (B): a) overview of the cell monolayer at the magnifications of 400× for control cells and 1000× for DMSO cryopreserved cells; b) typical thick carpet pattern of brush-border organization (10000×); c) close view of a cluster of microvilli on the cell surface (40000×).

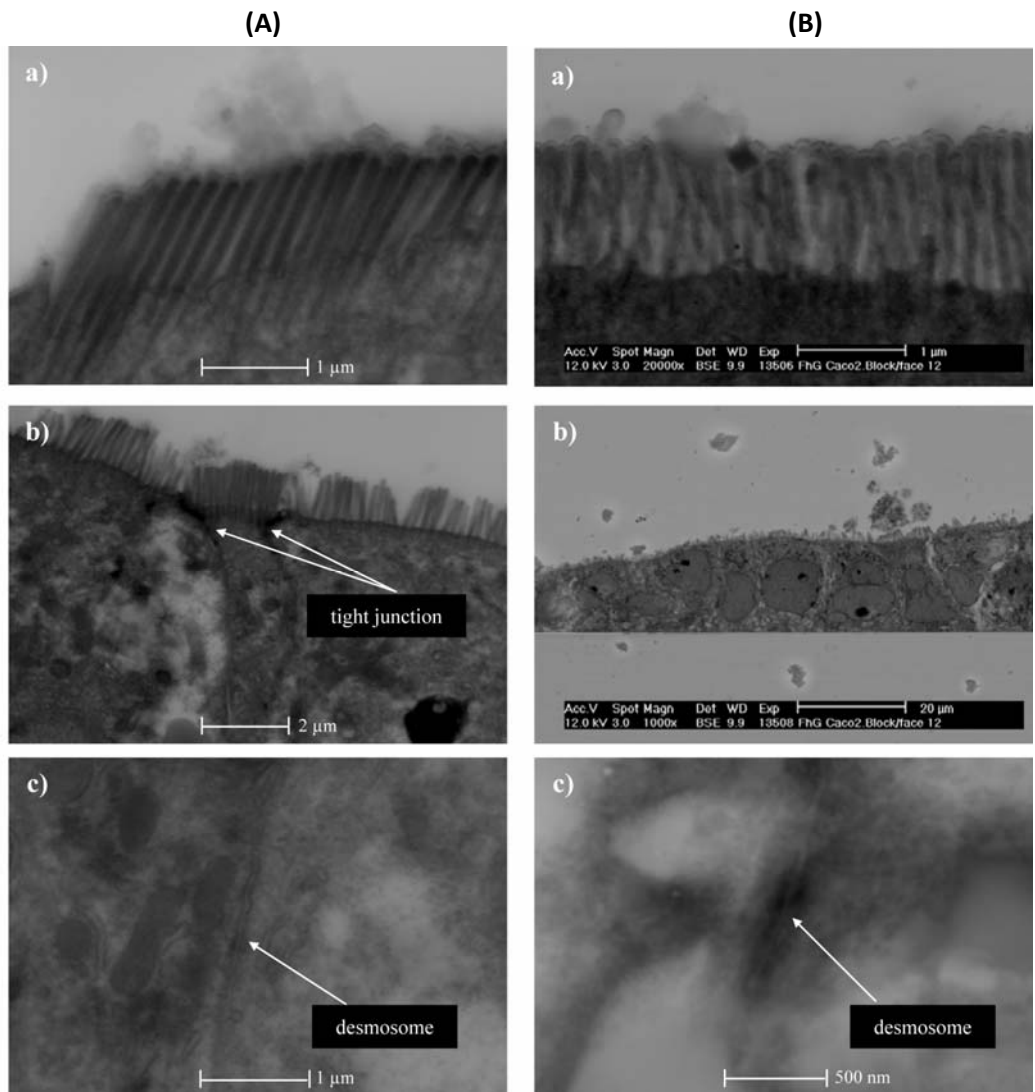


Figure 5. Block-face-SEM of a cross-section of a monolayer of Caco-2 cells after 17 days in culture. The typical features of Caco-2 cells differentiation are shown for both control cells (A) and cells cryopreserved in micro-cryosubstrates with 5% DMSO before culture (B): a) brush border microvilli projecting perpendicular to the surface at the magnifications of 20000 \times for control cells and 20000 \times for DMSO cryopreserved cells; b) tight junction (8000 \times for control cells) and cross section of the monolayer showing the brush border microvilli only at the apical surface of the cells in contact with the medium (13500 \times for DMSO cryopreserved cells); c) desmosome at magnifications of 20000 \times for control cells and 40000 \times for DMSO cryopreserved cells.

Both Caco-2 cells which had been frozen with 5% DMSO in micro-cryosubstrates and control cells were covered by typical brush border microvilli

projecting perpendicular to the surface (Figures 4 and 5). SEM observations revealed that the brush border microvilli covered the whole surface of the cell, exhibiting a typical thick carpet distribution pattern (Figures 4Aa, 4Ba and 4Bb). Block-face-SEM of cross sections of the Caco-2 monolayers has also confirmed the morphological differentiation of both cells cryopreserved with 5% DMSO in micro-cryosubstrates and control cells: polarization of the cells is characterized by their asymmetry (brush border microvilli were located only at the apical surface) (Figure 5Bb) and by the junctional complexes which were composed of tight junctions (Figure 5Ab) and desmosomes (Figures 5Ac and 5Bc).

4. DISCUSSION

The use of miniaturised cryosubstrates may be an intelligent solution for the storage of small volumes of a large number of samples as already shown for the storage of the established L929 fibroblast cell (Zimmermann et al. 2003). In the study reported herein, the micro-cryosubstrates were compared to the traditionally used 1 ml cryovials, in order to evaluate the effect of miniaturisation on the cell viability and function; the cell model used was the Caco-2. For this purpose two different permeable CPAs, DMSO and glycerol, and two different processes for CPA addition to the micro-cryosubstrates, manually or using an automatic pipette robot system, were tested.

Although being the most commonly used CPA for cell storage, several problems have been associated with the use of DMSO, such as cytotoxicity, effects on cell cycle and apoptosis (Davis et al. 1990; Baum et al. 1992; Santos et al. 2003; Syme et al. 2004) and contribution to the undirected differentiation of stem cells upon thawing (Ji et al. 2004). Thus the reduction or complete replacement of DMSO by alternative, less toxic, medically approved substances is an interesting option.

CPAs are employed to reduce cell damage following freezing and thawing. Penetrating CPAs, such as DMSO and glycerol, achieve their protective effects by permeating the cells and helping to maintain an osmotic balance between the intra- and extracellular environment when cells are attempting to re-equilibrate the solute and ionic concentrations in the cytoplasm with extracellular concentrations during the freezing process (Lovelock 1953a; Lovelock 1954; Lovelock et al. 1959; McGann 1978; McGann 1979). For glycerol, however, the mechanism of action has shown to be more complex, since it is in fact a penetrating agent if added at physiological temperatures, but it is essentially non-permeant if added at 0°C (Mazur et al. 1976; McGann 1979; Frim et al. 1983). Our results show that, for the freezing conditions used, DMSO was more effective than glycerol in maintaining high post-thaw Caco-2 cells viability, using either micro-cryosubstrates or cryovials, especially for the lower concentrations tested (5% and 3%). The lower viability percentages obtained for cells frozen with glycerol when compared to DMSO may be related to the fact that at 4°C glycerol was not able to permeate the cells and perform its protective action. On the other hand, even if it is able to effectively permeate Caco-2 cells at that temperature, it has been shown that for most cells glycerol is a slower permeating molecule than DMSO (Gilmore et al. 1998; Xu et al. 2003). If this is true for Caco-2 cells, then DMSO may have been more effective in reducing cell osmotic stress the cells since it could penetrate the cells faster, thus replacing intracellular water more rapidly. The lower permeability of glycerol may also explain the difference on cell viability found for micro-cryosubstrates filled manually or automatically. When DMSO was used, no difference was found on the post-thaw viability of cells frozen in micro-cryosubstrates, either manually or automatically filled for all the concentrations tested. However, when glycerol was used, filling the micro-cryosubstrates with the pipette robot system “Nanoplotter” lead to a considerably increase on cell viability values compared to freezing in manually filled micro-cryosubstrates. This may be due to a more immediate and homogeneous distribution of the CPA in the

freezing medium when the automatic system is used, allowing a faster penetration of glycerol into the cells.

It is also important to notice that, as freezing injury to cells is dependent on the interaction of the major cryobiological variables, CPA type and concentration and cooling and warming rates (Leibo et al. 1970), and it has been shown that glycerol's CPA action will significantly vary with cooling and warming rates (Morris et al. 1972), it is possible that the 1°C/min rate used to freeze the cells was not the optimum rate for the glycerol concentrations used.

Cell damage during freezing and thawing has multiple causes, e.g. it has been associated with loss of plasma membrane integrity (Dankberg et al. 1976; Morris 1981). Although the exact mechanisms of freezing injury can not be predicted for a single cell, the main effects and cell reactions during the process are known. During the freezing-thawing process cells are exposed to several stresses, such as increasing solute and ionic extracellular concentrations, salt precipitation and alterations in pH and viscosity, which may cause changes to the more sensitive components or structures within the cell. The cells respond to these conditions by different mechanisms, such as osmotic shrinkage, membrane lipid phase changes, solubilisation of membrane proteins, destabilisation of the lipid bilayers; mechanical damage to membranes and the cytoskeleton and denaturation of proteins may also take place (Mazur 1970; Mazur 1984; Mazur et al. 1986). Therefore, cells with intact plasma membrane, which are assumed to be viable, may not be able to fully recover their function after thawing and efficiently resume growth and metabolic activity, being critical to assess the cells behaviour during long-time culture after thawing, namely concerning their proliferation and differentiation capacity.

Proliferation tests were performed with cells which had been previously cryopreserved in micro-cryosubstrates or cryovials. Cells cryopreserved with 10% or 5% DMSO, in either micro-cryosubstrates or cryovials, were able to proliferate and grow at rates similar to cells which had not been frozen. However, in the case

of cells which have been cryopreserved with 10% glycerol in micro-cryosubstrates, the proliferation rates were lower than for control cells. One hypothesis is that, although many of the cells were in fact able to start proliferating at the same rate as control cells, there was a certain percentage of cells which did not survive when inoculated after thawing, leading to a lower number of proliferating cells in culture. This last hypothesis is in conformity with microscopic observations of many dead cells, mainly during the first 3 days of culture. This difference between the number of viable cells measured immediately after thawing and the ones that do in fact survive the freeze-thaw cycle and attach to the culture surface and continue the cell cycle for several generations has been first reported in 1978 (Frim et al. 1978) and it is also in agreement with more recent studies reported by other authors, describing the activation of the apoptotic pathway as a consequence of cryopreservation, leading to a decline in cell viability and function, mainly in the first 24 hours after cell thawing (Katsen et al. 1998; Baust et al. 2002; Baust 2005).

Aiming for a cryopreservation process that allows for cells to initiate immediately their specific function after thawing so they can be used for therapeutic purposes, it is critical to check their differentiation capacity after thawing. The Caco-2 cell line was used due to its robustness, well established characterization and easy assessment of cell differentiation after long-time culture. Post-confluent monolayers of Caco-2 cells are very similar to mature enterocytes with regard to their functional and morphological characteristics (Chantret et al. 1988; Delie et al. 1997; Pageot et al. 2000). During Caco-2 cells spontaneous differentiation into mature enterocytes, brush border membrane enzymes, such as sucrase-isomaltase, aminopeptidase and alkaline phosphatase, are expressed at higher levels after differentiation is achieved (Pinto 1983; Chantret et al. 1988). SEM studies revealed that differentiated Caco-2 cell layers are covered by typical brush-border microvilli, characteristic of cell polarization (Pinto 1983). Observation by transmission electron microscope (TEM) also

revealed typical brush border microvilli as well as the presence of junctional complexes constituted by tight junctions, which separate basolateral and apical domains, as well as desmosomes (Pinto 1983)

In this work, the expression of the brush border enzyme alkaline phosphatase was used as a marker of cell differentiation. Cells which had been cryopreserved with 10% or 5% DMSO, in either micro-cryosubstrates or cryovials, were able to express alkaline phosphatase at the same rate as control cells. The activity of this enzyme started to increase gradually after confluence, as has been described by other authors (Pinto 1983; Delie et al. 1997). The highest enzyme activity was obtained at the 22nd day of culture, corresponding to the last day of the assay.

For cells which had been cryopreserved with 10% glycerol the activity of alkaline phosphatase during growth was lower than for control cells, although following the same profile; this is in agreement with the results of the proliferation tests.

The inverted backscattered electron imaging of block-face preparations allowed the observation of cell-cell contacts giving simultaneously information on cell ultrastructure. SEM and block-face SEM images show the apical surface of cells coated with a highly ordered, dense array of microvilli. The block-face preparations also show cell features such as tight junctions and desmosomes typical for these cells. No difference was found on the morphology of cells which had been cryopreserved with 5% DMSO in micro-cryosubstrates and control cells.

As shown both by the measurement of the brush border enzyme alkaline phosphatase as by SEM and block-face SEM observations, cells cryopreserved in micro-cryosubstrates using DMSO were able to recover quickly from freezing and start to differentiate in the same way as control cells and cells frozen in cryovials, whereas cells cryopreserved with glycerol in micro-cryosubstrates took a longer time to recover from the freezing process and start differentiating.

Together with the results obtained for post-thaw membrane integrity and proliferation tests, the overall conclusion is that DMSO ensured Caco-2 cells fully recover of proliferation and differentiation capacities after thawing, using either micro-cryo-substrates or 1 ml cryovials. Furthermore, a lower concentration (5% or even 3%) than the usually recommended (10%) can be used, which might be advantageous given some cytotoxic systemic side effects associated to DMSO.

5. CONCLUSIONS

The results presented herein show that it is possible to efficiently cryopreserve Caco-2 cells in suspension using micro-cryosubstrates. The significant reduction of the freezing volume from 1 ml to 25 μ l did not affect the post-thawing cell membrane integrity neither cell function nor differentiation potential during long-time culture after thawing in contrast to results achieved with L929 fibroblast (Zimmermann et al. 2003). The ability of DMSO on maintaining high post-thaw cell viability, suggested by membrane integrity tests, was confirmed by the proliferation and differentiation studies. Although the complete replacement of DMSO by the non-toxic agent glycerol has shown not to be advantageous for Caco-2 cells cryopreservation, the results presented herein indicate that it is possible to reduce the DMSO concentration from 10% to 5%. Moreover, the results from the cell membrane integrity assay show that thus we might extrapolate that this concentration may also be used for cryopreservation of Caco-2 cells allowing high proliferation and differentiation rates post-thawing.

Finally, an efficient novel strategy for cell cryopreservation, based on miniaturisation, automation and sample information, was validated, which is of major importance on the establishment of large cell banks. This cryopreservation system did not affect cells capacity to proliferate and differentiate a fundamental requirement for further cell-therapeutic utilizations.

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CRYOPRESERVATION OF 2-D MONOLAYERS

Cryopreservation of Adherent Cells: Strategies to Improve Cell Viability and Function after Thawing

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ABSTRACT

The commonly applied cryopreservation protocols routinely used in laboratories worldwide were developed for simple cell suspensions and their application to complex systems, such as cell monolayers, tissues or biosynthetic constructs, is not straightforward. In particular for monolayer cultures, cell detachment and membrane damage are often observed after cryopreservation.

In this work, combined strategies for the cryopreservation of cells attached to matrigel-coated well-plate's surfaces were investigated based on cell entrapment in clinical grade, ultra-high viscous alginate using two cell lines, neuroblastoma N2a and colon adenocarcinoma Caco-2, with distinct structural and functional characteristics. As the cryopreservation medium, serum-free CryoStorTM solution was compared with serum-supplemented culture medium, both containing 10% dimethyl sulfoxide (DMSO).

Using culture medium, entrapment beneath an alginate layer was needed to improve cell recovery by minimizing membrane damage and cell detachment after thawing; nevertheless, up to 50% cell death still occurred within 24 hours after thawing. The use of CryoStorTM solution represented a considerable improvement of the cryopreservation process for both cells lines, allowing the maintenance of high post-thaw membrane integrity as well as full recovery of metabolic activity and differentiation capacity within 24 hours post-thawing; in this case, entrapment beneath an alginate layer did not confer further protection to cryopreserved Caco-2 cells but was crucial for maintenance of attachment and integrity of N2a neuronal networks.

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1. INTRODUCTION

Cryopreservation provides great benefits to a wide variety of mammalian cell-based applications including reproductive embryology, regenerative and reparative medicine and biomedical research, as well as to *in vitro* drug screening and toxicology testing. Such benefits can only be fully accomplished if cell function is maintained during the cryopreservation process; this can be critical in the case of complex and sensitive systems such as embryonic stem cells, engineered cells and tissues, monolayer cell cultures, 3-dimensional cell aggregates or biosynthetic constructs (Karlsson et al. 1996; Reubinoff et al. 2001; Baust et al. 2006).

The commonly applied protocols for cryopreservation of cell suspensions consist of slow cooling and rapid warming rates in cell culture media containing high concentrations of DMSO and animal-derived products. It does not take into account the differences between complex cell-systems and simple cell suspensions with respect to their responses to cooling, warming and dehydration, which often results in poor post-preservation quality (Baust et al. 2006). Thus, while many cells in suspension can be cryopreserved with almost complete recovery, it has been demonstrated that cells in monolayers are often more susceptible to cryoinjury (Berger et al. 1996; Hornung et al. 1996; Zieger et al. 1996; Acker et al. 1999; Pasch et al. 1999; Ebertz et al. 2004). Cell-cell and cell-matrix junctions and the organization of the cytoskeleton have been shown to render cells more sensitive to cryoinjury when compared to isolated cells in suspension (Armitage et al. 1996; Acker et al. 1999). Furthermore, the close interactions between cells are potential sites for damage caused by the osmotic stresses and phase changes involved in the process of cryopreservation (Armitage et al. 1995). Finally, there appears to be an enhanced susceptibility to intracellular ice formation (IIF) in cells adherent to a substrate when compared to cells in suspension, even in the absence of cell-cell interactions (Mazur 1965; Berger et al.

1996; Acker et al. 2001), although the exact relation between IFF and cryoinjury in adherent cells is still unclear (Acker et al. 2002).

Successful cryopreservation of ready-to-use cell monolayers would support the implementation of routine cryopreservation practices during preparation of engineered cells and tissues for clinical applications (Baust 2005) and benefit high-throughput cell-based assays by reducing batch-to-batch variation and eliminating the time consuming, labor intensive process of inoculation and expansion from a frozen vial of cells, thus reducing time between cell storage and use in experimental settings.

Different cryopreservation methodologies alternative to the commonly applied protocols are reported in the literature, namely cell entrapment in a gel or ECM has been proposed as a promising cryopreservation strategy (Koebe et al. 1990; Hubel et al. 1991; Ruoslahti et al. 1997; Koebe et al. 1999; Ji et al. 2004). Amongst the biopolymers used in biotechnological and medical applications, clinical-grade, highly purified alginate of extremely high viscosity extracted by a novel process and uniformly cross-linked with Ba^{2+} has been described as an improvement for the entrapment of cells, tissues or other biologically active material (Zimmermann et al. 2005).

In this study, a novel cryopreservation strategy based on cell entrapment beneath a layer of clinical grade, ultra-high viscosity permeable alginate hydrogel was investigated. Two cell lines, mouse neuroblastoma N2a and human colon adenocarcinoma Caco-2, were used as model systems due to their distinct structural and functional characteristics as well as cell-cell and cell-matrix interactions.

Caco-2 cells, after long-term culture (15-25 days), spontaneously differentiate to cells expressing morphological and functional characteristics of the mature intestinal enterocytes (Rousset 1986; Delie et al. 1997), which renders them valuable *in vitro* models and high-throughput screening tools in the pharmaceutical industry (Blais et al. 1987; Shah et al. 2006). Neuroblastoma cells

are able to differentiate into randomly connected neuronal populations through treatment with retinoic acid, which has promoted their extensive use as model systems to study neuronal communications and screen novel compounds for neurotoxic properties (Thiele 1998; Tropepe et al. 1999; Bani-Yaghoub et al. 2005; LePage et al. 2005). Successful cryopreservation of ready-to-use micro-plates of both cell lines would reduce heterogeneity of sub-populations, thus promoting the standardization of these models and data comparison from different laboratories, and support the implementation of faster drug screening assays (Sambuy et al. 2005; Zucco et al. 2005).

The use of standard cell culture media was compared with protein- and serum-free, “intracellular-like” (low Na^+ , high K^+), CryoStor™ solution as the vehicle solution for the cryoprotectant (CPA) (DMSO) as it has been reported to significantly enhance post-thaw cell survival and recovery in a series of cellular systems (Elford et al. 1972; Baust et al. 2001; Baust et al. 2002; Baust et al. 2002; Sosef et al. 2005; Baust et al. 2006; Stylianou et al. 2006).

2. MATERIALS AND METHODS

2.1. MATERIALS

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA (1x solution) and alamarBlue were obtained from Invitrogen (Carlsbad, CA). RPMI-1640 medium, poly-L-lysine (PLL), poly-D-lysine (PDL), retinoic acid (RA), DMSO, fluoresceine diacetate and ethidium bromide, were obtained from Sigma (Steinheim, Germany). Culture plastic-ware was obtained from Nunc (Roskilde, Denmark). Matrigel was obtained from BD Biosciences (San Jose, CA, USA). CryoStor™-CS10 was obtained from BioLife Solutions (Bothell, WA, USA). Other chemicals were of the purest grade available from regular commercial sources.

Alginates: Ultra-high viscous (UHV) alginates (viscosity of a 0.1% (w/v) solution in distilled water was 20-30 mPa.s) were produced and purified from stipes of *L. nigrescens* and *L. trabeculata* as described elsewhere (Zimmermann et al. 2001; Leinfelder et al. 2003; Wolf et al. 2005; Zimmermann et al. 2005). A 1:1 mixture of purified alginates was used at 0.7% (w/v) in NaCl..

2.2. CELL CULTURE

The Caco-2 cell line was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat inactivated FBS and sub-cultured every 3-5 days. Differentiation into enterocyte-like cells was achieved by long time culturing (up to 21 days) with medium exchange every 3-4 days.

The N2a cell line was obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were maintained in DMEM supplemented with 10% (v/v) heat inactivated FBS and 4.5g/l glucose and sub-cultured every 5 days. For induction of differentiation, the medium was replaced by DMEM + 2% FBS + 15 μ M RA 24 hr post-plating and changed every 2 days during 5 days.

Both cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. All experiments were performed using Caco-2 and N2a cells between passages 10 to 30.

2.3. CELL CULTURE BENEATH AN UHV ALGINATE GEL LAYER

Caco-2 and N2a cells were seeded onto sterilized glass coverslips (Marienfeld GmbH, Germany) coated with matrigel (0.28 mg/ml in culture medium), inside 4-well plates, at a density of 1×10^4 and 5×10^3 cells/cm², respectively, and cultured for 5 days in the respective growth medium or differentiated (as described above).

An UHV alginate gel layer was added on top of the cells either on day 1 or 4 post-inoculation in order to assess the best addition time for alginate. Cells were rinsed in phosphate-buffered saline (PBS), incubated with PLL for 30 min (to allow adhesion of alginate to the well's surface) and washed with PBS before addition of 100 μ l/well alginate gel (approximately 2.5 μ m thickness, as estimated through electron microscopy). Alginate gelling was achieved through 25 min incubation with 20 mM BaCl₂ solution followed by three times washing with PBS with 0.5 mM MgCl₂.

Cells cultured in the same conditions without an alginate layer were used as control.

2.4. CELL CRYOPRESERVATION

Undifferentiated Caco-2 and N2a cells were frozen after 5 days of culture inside the 4-well plates with 300 μ l/well cryopreservation solution, which consisted of either culture medium supplemented with 10% DMSO or CryoStorTM-CS10. Differentiated N2a and Caco-2 cells were similarly cryopreserved (after 5 and 21 days of culture, respectively) in CryoStorTM-CS10. Cells were allowed to equilibrate in the cryopreservation solution for 30 min at 4°C and cooled to -80°C, at a cooling rate of 1°C/min, using a rate-controlled freezing system (Planer Kryo 560-16, Planer, Middlesex, UK). The plates were stored at -80°C for 1-2 weeks. Following storage, cells were thawed in a CO₂ incubator (37°C, 5% CO₂ in air) (approximately 15°C/min warming rate) and a one-step dilution (1:3) in growth medium was performed. Total growth medium replenishment was performed 30 min after and cells were further cultured for post-thaw studies or used for membrane integrity assays immediately after thawing. The UHV alginate layer remained on top of the cells during further culture and recovery assays.

2.5. CELL RECOVERY ASSAYS

Cell viability was assessed before freezing and after thawing daily for 3 days, both through a qualitative cell membrane integrity assay and the quantitative alamarBlue assay. Cell growth and recovery after thawing was also monitored during 3 days by visualization on an inverted microscope (Leica DM IRB).

2.5.1. Cell Membrane Integrity Assay

The enzyme substrate fluorescein diacetate (FDA) and the DNA-dye ethidium bromide (EB) were used as described in the literature (Malpique et al. 2007).

2.5.2. AlamarBlue Assay

The metabolic indicator alamarBlue was used following the manufacture's recommendation. Briefly, cells were incubated with fresh medium containing 10% alamarBlue and 4 hours later fluorescence was measured in 96-well plates using a microplate fluorescence reader (Biotek Instruments, Winooski, VT, USA).

2.5.3. Cell Differentiation Assessment

2.5.3.1. Caco-2 Differentiation

The differentiation state of post-confluent Caco-2 monolayers before and after cryopreservation as well as the capacity of confluent (5 days culture) Caco-2 cells to spontaneously differentiate during long-term post-thaw culture were evaluated by quantifying the activity of the brush-border enzyme alkaline phosphatase. This was performed by measuring the reaction of the enzyme with the substrate 4-methylumbelliferyl phosphate (4-MUP) as described in the literature (Malpique et al. 2007).

2.5.3.2. N2a Differentiation

Neuronal differentiation was assessed before and after cryopreservation using an immunocytochemistry assay with neuronal cell markers. For cells covered with an alginate layer, this was dissolved before performing the assay by incubating with a 20 mM Na₂SO₄ solution for 20 min at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells adherent to glass coverslips were processed as described previously (Serra et al. 2007).

Primary antibodies and dilutions used were: anti-tubulin beta III isoform (β -tubulin, 1:10; Chemicon, Temecula, CA, USA), anti-microtubule-associated protein-2 (MAP2a/b, 1:100; Sigma Steinheim, Germany). The secondary antibodies and dilutions used were: anti-mouse Alexa 488 (1:500) and anti-rabbit Alexa 594 (1:500) (Molecular Probes, Eugene, OR, USA). Cells were visualized in a Leica DMRB fluorescence microscope (Leica Microsystems GmbH Wetzlar, Germany)

2.5.4. Scanning Electron Microscopy

To evaluate the structural integrity and differentiation state of Caco-2 monolayers, scanning electron microscopy (SEM) was performed before and after cryopreservation. Cells adherent to the glass coverslips were prepared as described elsewhere (Katsen et al. 1998) and SEM studies were performed on field emission scanning electron microscope type FESEM XL30 (Phillips, USA).

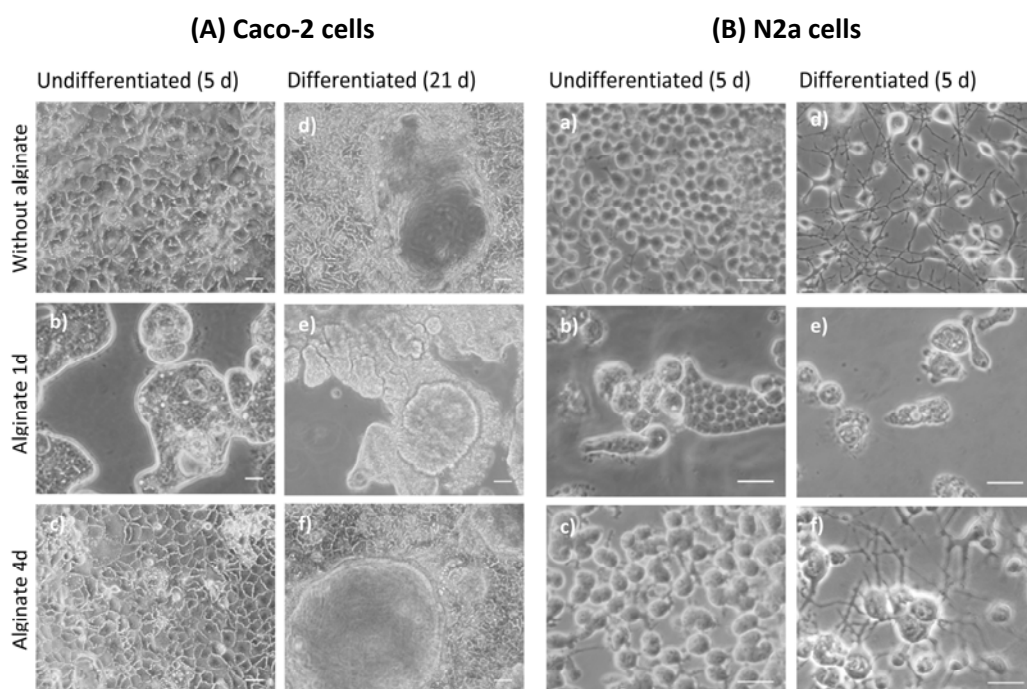
2.5.5. Data Presentation/Analysis

Each experimental condition represents $n \geq 6$ experimental replicates (minimum of 2 experimental repeats with 3 sample replicates per experiment. Error bars denote the standard deviation of the mean. Comparison of mean values between groups was performed using analysis of variance (ANOVA), with $P < 0.05$ considered statistically significant. Representative photographs are depicted.

3. RESULTS

3.1. EFFECT OF UHV ALGINATE ENTRAPMENT ON CELL GROWTH, MORPHOLOGY AND DIFFERENTIATION

Cell entrapment beneath a layer of alginate gel added 1 or 4 days post-inoculation, resulted in different responses to growth and differentiation of both cell lines as shown in Figure 1.



(C)

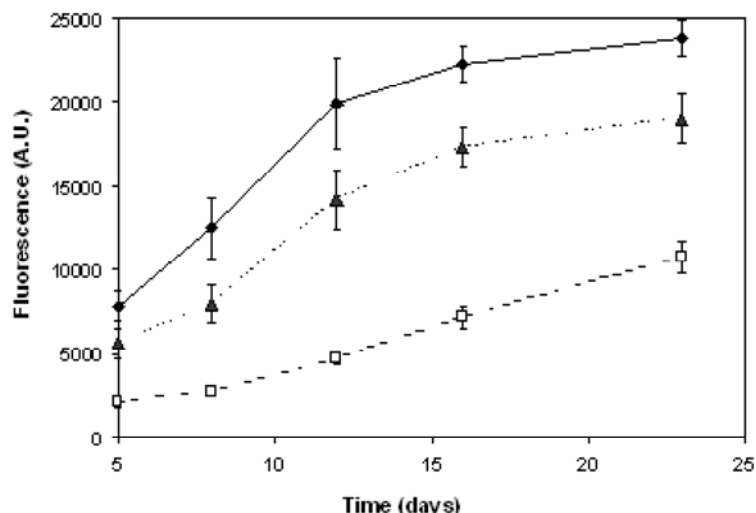


Figure 1. Effect of alginate entrapment on Caco-2 and N2a cell cultures on 4-well plates. A and B: Phase-contrast photographs of Caco-2 (A) and N2a cells (B) in an undifferentiated state after 5 days of culture (a, b and c) or differentiated after 21 and 5 days of culture (d, e and f) for Caco-2 and N2a cells, respectively. Scale bars = 50 μ m. C: AlamarBlue fluorescence during culture without alginate (black circles) or beneath a layer of alginate added 1 (white squares) or 4 (black triangles) days post-inoculation. A.U. = Arbitrary units, Alginate 1d/4d = Alginate added 1 or 4 days after cell inoculation.

Control cultures (without alginate) of Caco-2 and N2a cells formed confluent monolayers after 4 days in culture (Figures 1Aa and 1Ba). With alginate added 1 day post-inoculation, both cell lines lost their characteristic shape and grew beneath the alginate layer as closely packed cell aggregates displaying round cell morphology (Figures 1Ab and 1Bb). Furthermore, Caco-2 cells proliferated at lower rates when compared to control cultures as confirmed through alamarBlue assays (Figure 1C). Whenever the alginate was added 4 days post-inoculation, Caco-2 cells capacity to proliferate and differentiate during long-term culture was not affected as shown by metabolic activity profiles (Fig. 1C) and the development of the morphological features typical of enterocyte differentiation, such as domes and flower-like clusters on the cell surface (Figure 1Af). The difference in fluorescence values observed between control and alginate-covered cells (Figure 1C) might be due to a delayed diffusion of the metabolic products through the

alginate matrix leading to the incomplete alamarBlue reaction. After alginate dissolution, no significant difference was found between control and alginate-entrapped cells confirming that alginate entrapment 4 days post-inoculation does not affect cell growth (data not shown).

Concerning N2a cells neuronal differentiation, whereas alginate addition at day 1 resulted in significant reduction of neurite sprouting (Fig. 1Be) as well as inhibition of neuronal markers expression (data not shown), adding alginate 4 days post-inoculation did not affect the morphology of the neuronal networks (Figs. 1Bf) nor the expression of typical neuronal markers as confirmed through immunocytochemistry (data not shown).

3.2. EFFECT OF UHV ALGINATE ENTRAPMENT AND CRYOMEDIUM ON POST-THAW RECOVERY AND MONOLAYER INTEGRITY OF UNDIFFERENTIATED CELLS

Cryopreservation efficacy was evaluated by cell recovery post-thawing, assessed by the alamarBlue assay (Figure 2) as previously described (Mathew et al. 1999; Pasch et al. 1999; Baust et al. 2000). Additionally, loss of membrane integrity and cell detachment from the surface after thawing were qualitatively evaluated using a dual fluorescent membrane integrity test (Figure 3).

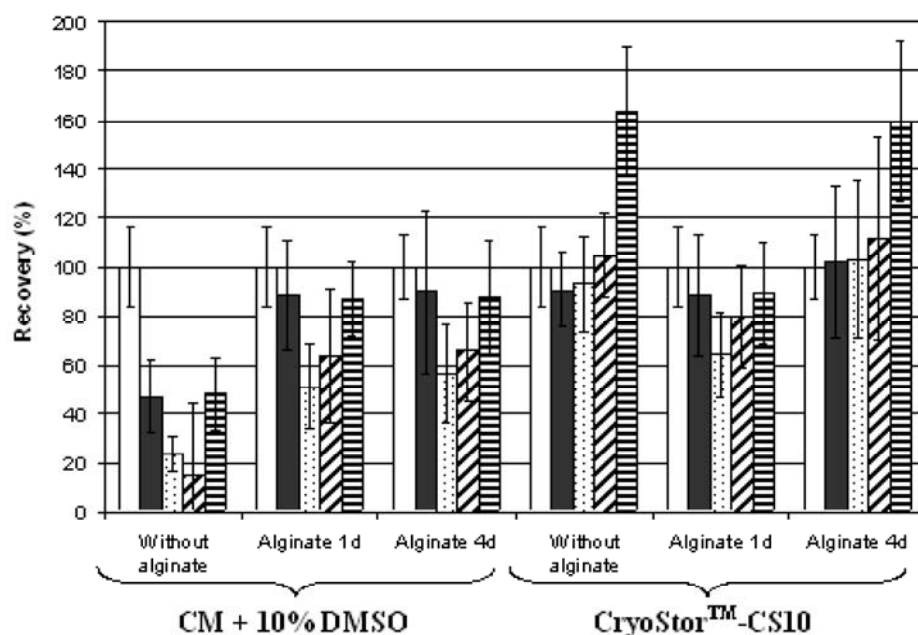
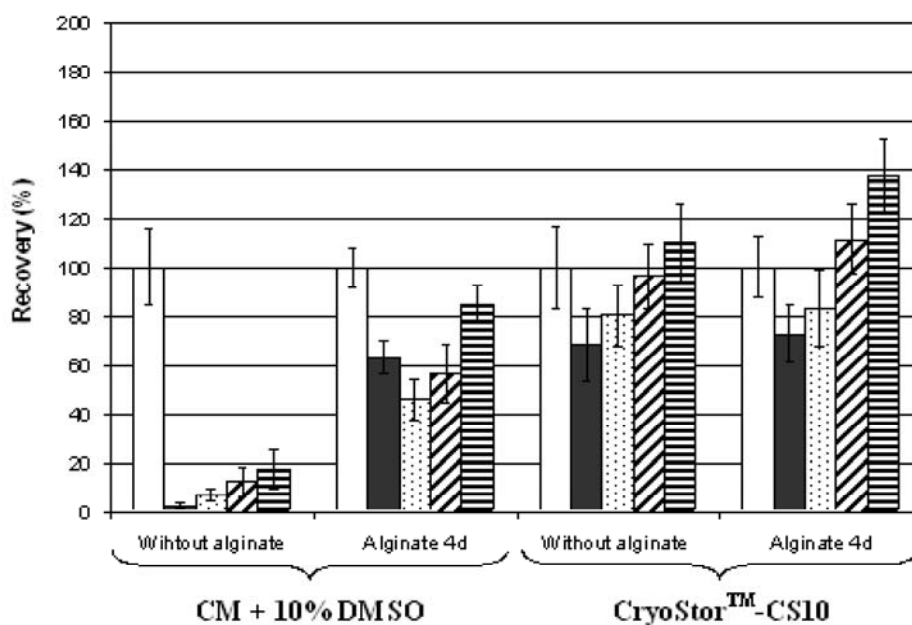
(A) Caco-2 cells**(B) N2a cells**

Figure 2. Effect of cryopreservation medium and alginate entrapment on the recovery of Caco-2 (A) and N2a (B) cells after cryopreservation. Both cell lines were cryopreserved after 5 days of culture in an undifferentiated state without alginate or beneath a layer of alginate added 1 or 4 days post-inoculation. The data represents alamarBlue fluorescence immediately (black bars), 1 (spotted bars), 2 (diagonal hatched bars) and 3 (horizontal hatched bars) days after thawing as a percentage of non-preserved control cells (white bars). CM = Culture medium, Alginate 1d/4d = Alginate added 1 or 4 days after cell inoculation.

Caco-2 cells

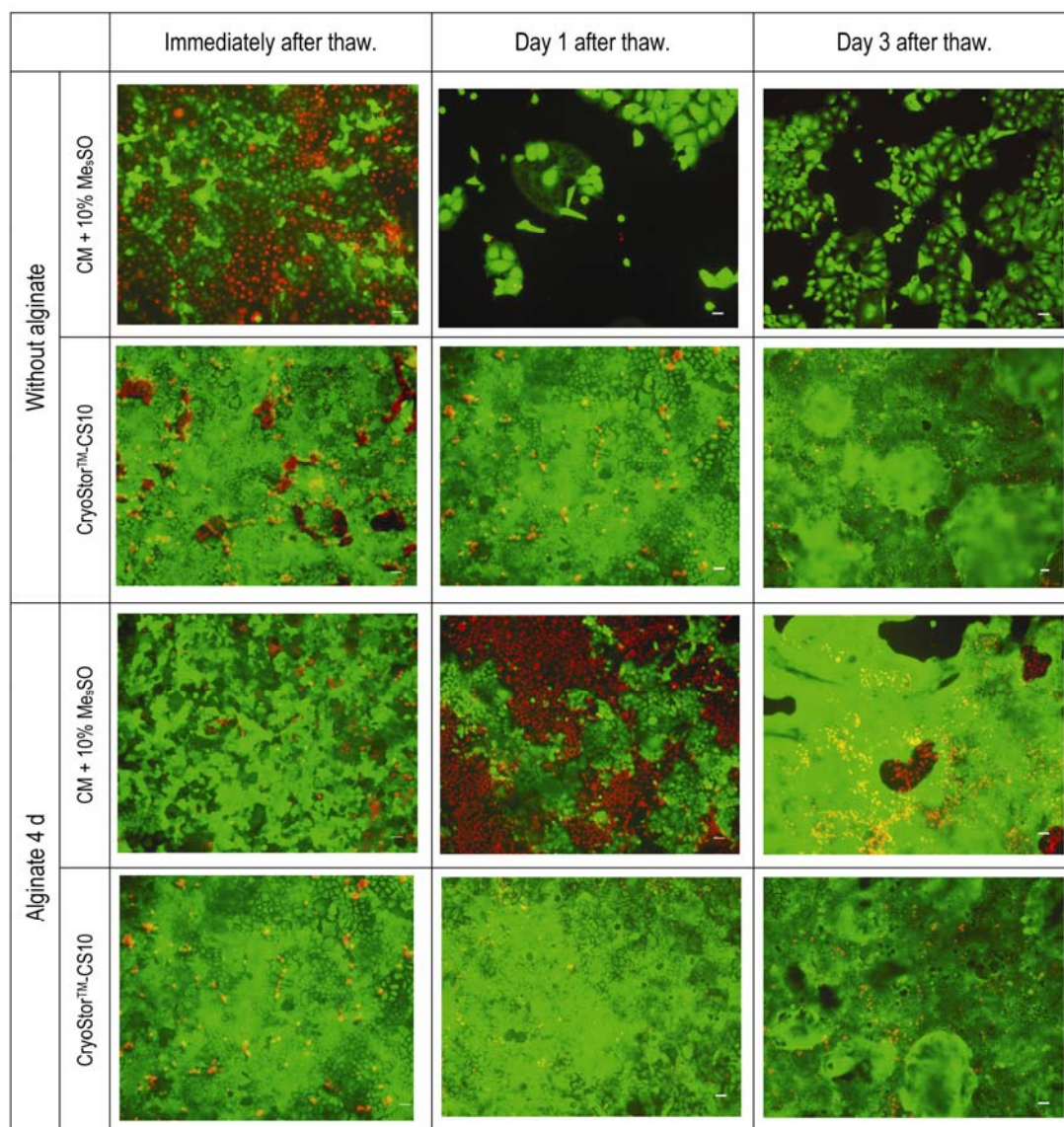


Figure 3. Effect of cryopreservation medium and alginate entrapment on the membrane integrity of Caco-2 cells after cryopreservation. Cells were cryopreserved in culture medium or CryoStor™-CS10 solution in an undifferentiated state after 5 days of culture without alginate or beneath a layer of alginate added 4 days post-inoculation, and assayed during 3 days after thawing with a dual fluorescence membrane integrity test: green cells with undamaged membranes and metabolic activity; red cells with damaged membranes. Scale bars = 50 μ m.

As shown in Figure 2, cryopreservation of both cell lines in culture medium without alginate led to a strong drop in metabolic activity immediately after thawing: $97\pm1\%$ and $53\pm14\%$ for N2a and Caco-2 cells, respectively. It is important to notice that such high loss of metabolic activity resulted from both damage of the cell membrane as well as high cell detachment from the well's surface shortly (within 30 min to 4 hours) after thawing (Figure 3), leading to cell loss when medium was exchanged during alamarBlue assays. Although the few cells that remained attached to the wells were able to recover and resume growth, only $17\pm8\%$ and $43\pm18\%$ recovery was found 3 days after thawing for N2a and Caco-2 cells, respectively.

Entrapment beneath a layer of UHV alginate added 4 days post-inoculation led to improved recovery of culture medium cryopreserved cells immediately after thawing ($90\pm33\%$ and $63\pm6\%$ for Caco-2 and N2a cells, respectively). This was due to reduction of both membrane damage and cell detachment after thawing, as shown in Figure 3 for Caco-2 cells. Despite such improvement, a 50% loss of cell viability still occurred within 24 hours post-thawing, when cells with damaged membranes were found beneath the alginate layer (Figure 3). Nevertheless, after 3 days in culture both cell lines were able to recover approximately 90% of the initial, i.e. before freezing, metabolic activity (Figure 2).

As depicted in Figures 2 and 3, the use of CryoStorTM-CS10 solution resulted in a considerable improvement of cell recovery immediately after thawing when compared to standard culture medium: $91\pm15\%$ and $68\pm15\%$ for Caco-2 and N2a cells, respectively. In this case, entrapment beneath an alginate layer added 4 days post-inoculation did not confer further protection to undifferentiated Caco-2 and N2a cells as high metabolic activity was found for both non-entrapped and alginate-entrapped cells immediately after thawing. Furthermore, full recovery of metabolic activity, initiation of proliferation and

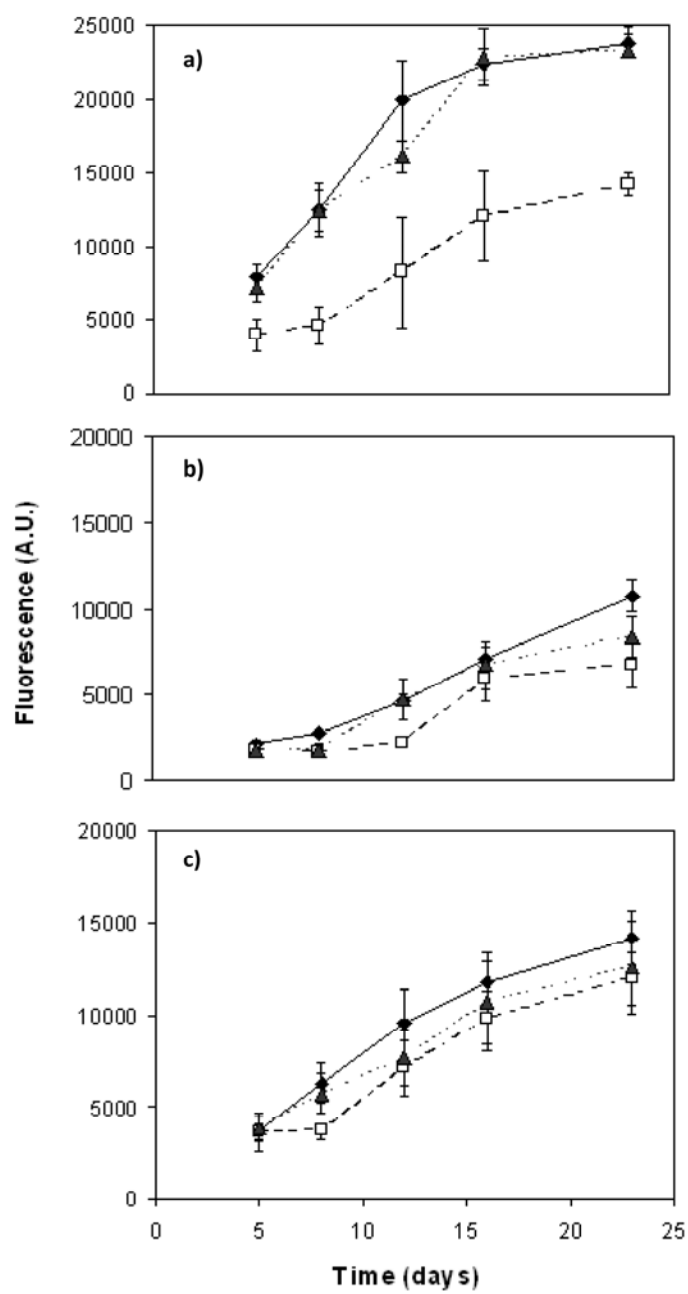
maintenance of cell membrane and monolayer integrity were observed within 24 hours post-thawing (Figures 2 and 3).

Concerning the moment of alginate entrapment, studies with Caco-2 cells revealed no significant difference on the immediate post-thaw recovery of cells cryopreserved beneath a layer of alginate added either 1 or 4 days post-inoculation: $88\pm23\%$ or $89\pm24\%$ and $90\pm33\%$ or $102\pm31\%$, for cells cryopreserved in culture medium and CryoStorTM-CS10, respectively. Thus, and since alginate entrapment 1 day post-inoculation led to a considerable inhibition of cell growth when compared to control cultures, alginate entrapment at day 4 was chosen for the continuation of the experimental work.

3.3. EFFECT OF CRYOPRESERVATION UPON PROLIFERATION AND DIFFERENTIATION CAPACITY

The capacity of Caco-2 cells to proliferate and differentiate when long-term (18 days) cultured after thawing is shown in Figures 4 and 5.

(A)



(B)

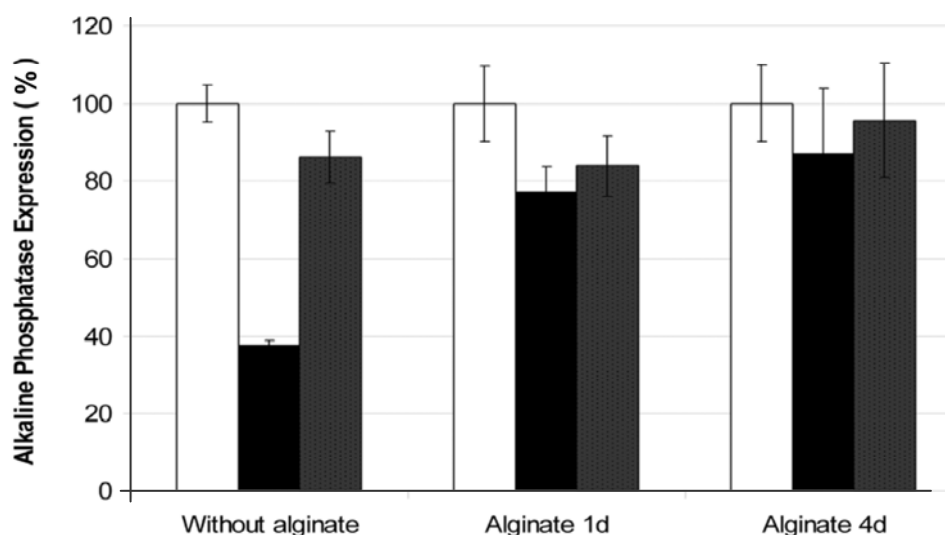
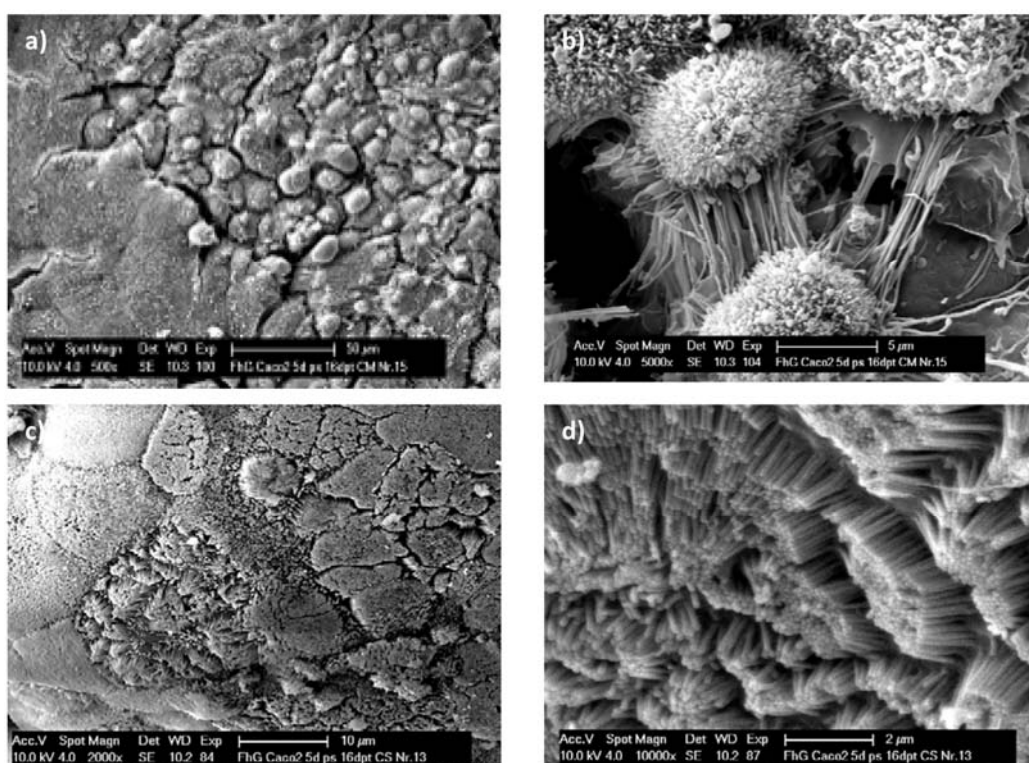


Figure 4. Effect of cryopreservation medium and alginate entrapment on (A) proliferation and (B) differentiation capacity of Caco-2 cells during long-term culture following cryopreservation. A: Cells were cryopreserved without alginate (a) or beneath an alginate gel layer added 1 day (b) or 4 days post-inoculation (c). Data represents alamarBlue fluorescence measurements during culture post- thawing for culture medium (white squares) or CryoStor™-CS10 (black triangles) cryopreserved cells and non-frozen control cells (black circles); B: Data represents alkaline phosphatase activity after 18 days of culture post-thawing for culture medium (black bars) or CryoStor™-CS10 (grey bars) cryopreserved cells as a percentage of the activity of non-frozen control cells (white bars). Alginate 1d/4d = Alginate added 1 or 4 days after cell inoculation.

One can observe that non-entrapped cells cryopreserved in culture medium were able to initiate growth only 3 days after thawing, which resulted in a significantly lower number of metabolically active cells in culture 18 days after thawing as compared to CryoStor™-CS10 cryopreserved and control cells (Figure 4Aa). When entrapped beneath a layer of alginate, added either 1 or 4 days post-inoculation, despite an initial delay in growth initiation, cells cryopreserved in culture medium were able to proliferate as both control cells and CryoStor™-CS10 cryopreserved cells; no significant difference was found in the number of metabolically active cells in culture after 18 days between the 3 samples (Figures 4Ab and 4Ac).

Concerning cell differentiation, as shown in Figure 4B, the percentage of alkaline phosphatase expressed after 18 days of culture post-thawing by CryoStor™-CS10 cryopreserved cells was comparable to the expression of this enzyme by control cells. No difference was found between cells cryopreserved with or without alginate entrapment. On the other hand, for cells cryopreserved in culture medium, alginate entrapment led to a considerable increase in the percentage of alkaline phosphatase expressed.



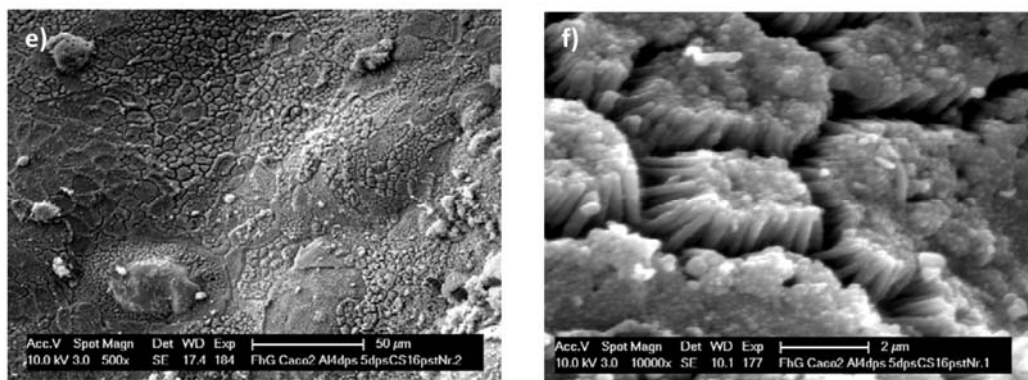


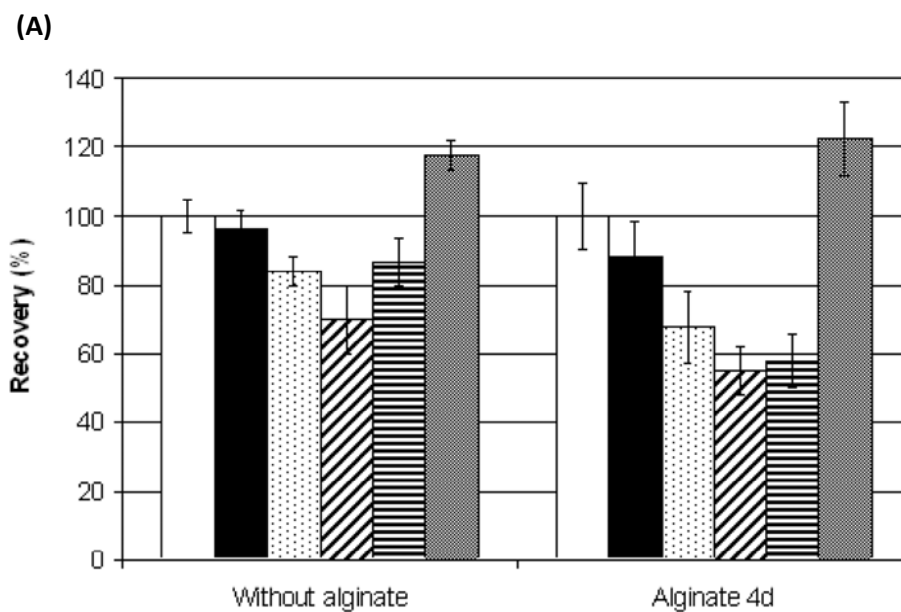
Figure 5. SEM photographs of Caco-2 monolayers after long-term culture (18 days) following cryopreservation in culture medium (a, b) or CryoStorTM-CS10 (c, d, e and f). Cells were cryopreserved without alginate (a, b, c and d) or beneath a layer of alginate added 4 days post-inoculation, which was dissolved after 18 days of culture post-thawing (e and f): a), c) and e) overview of the cell monolayer; b) close view of the cell junctions; d) and f) typical thick carpet pattern of brush-border organization with clusters of microvilli.

For cells cryopreserved in CryoStorTM-CS10, either with or without an alginate gel layer, SEM observations of Caco-2 cells cultured for 18 days post-thawing confirmed the high confluence, development of multiple cell layers and formation of a thick carpet of microvilli (Figures 5e, 5f and 5c, 5d). On the other hand, for cells cryopreserved in culture medium, full monolayer confluence could still not be observed and cell connections were still being formed after 18 days in culture (Figures 5a and 5b).

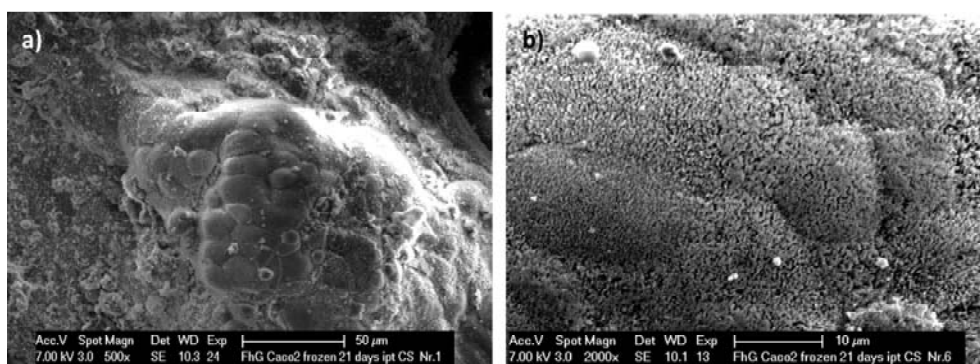
3.4. EFFECT OF UHV ALGINATE ENTRAPMENT AND CRYOMEDIUM ON POST-THAW RECOVERY AND MONOLAYER INTEGRITY OF DIFFERENTIATED CELLS

As confirmed by the results presented above, freezing cells in CryoStorTM-CS10 solution without alginate or beneath a layer of alginate added 4 days post-inoculation, resulted in higher cell recoveries post-thawing. These conditions were thus chosen for the cryopreservation of both cell lines in a fully differentiated state.

Caco-2 cells were frozen after 23 days of culture, which corresponded to fully differentiated cultures, forming multiple layers and covered by highly dense microvilli. Post-thaw evaluation of multi-layer integrity and cell survival was performed through membrane integrity tests (data not shown), assessment of metabolic activity and SEM analysis (Figure 6).



(B)



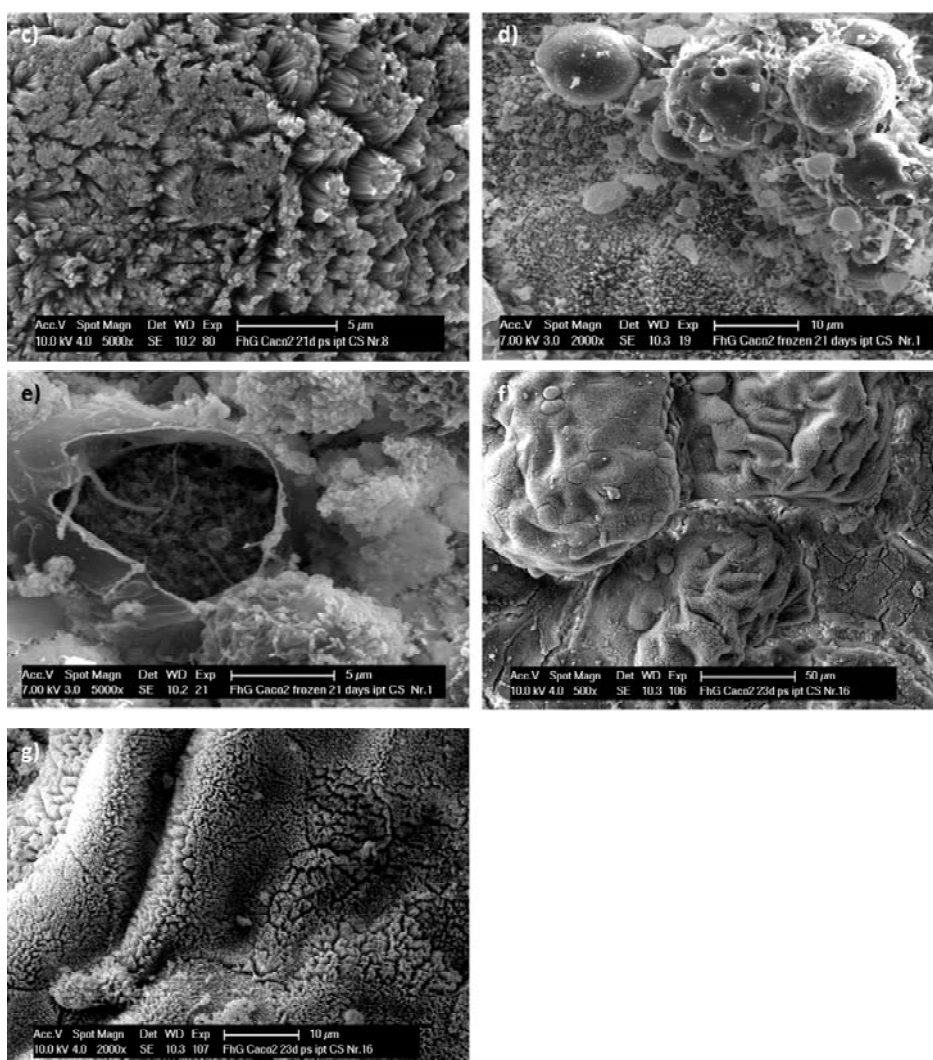
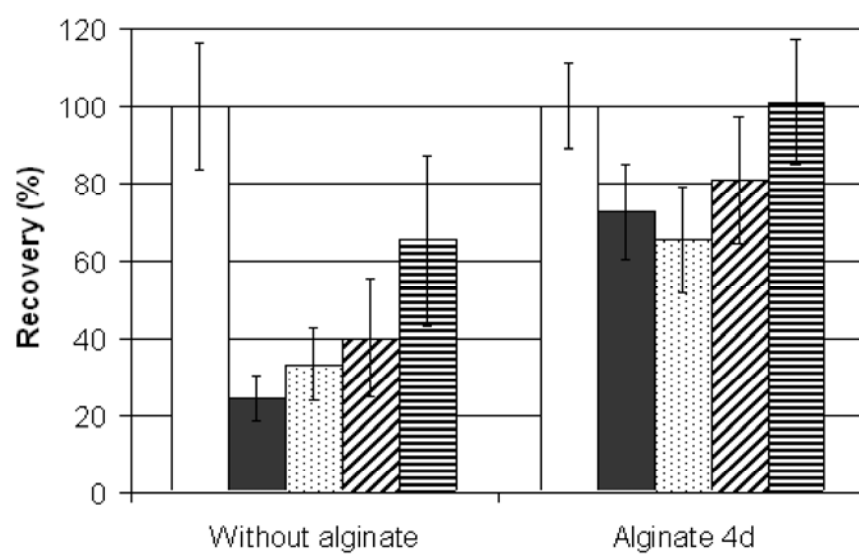
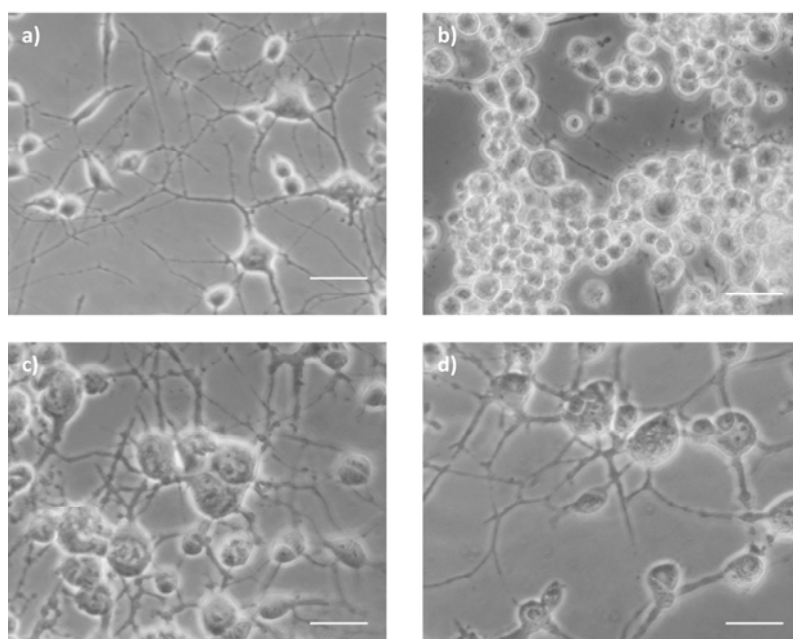


Figure 6. Effect of alginate entrapment on the recovery (A) and differentiation state (B) of differentiated Caco-2 monolayers after cryopreservation in CryoStor™-CS10. Cells were cryopreserved after 23 days of culture without alginate or beneath an alginate gel layer added 4 days post-inoculation. A: Data represents alamarBlue fluorescence immediately (black bars), 1 (spotted bars), 3 (diagonal hatched bars), 5 (horizontal hatched bars) and 10 (grey bars) days post-thawing as a percentage of non-preserved controls (white bars). Mean values were calculated from at least 6 well-plates thawed for each condition; B: SEM photographs, on aleatory microscopic fields within the same coverslip, of Caco-2 monolayers immediately after thawing: a), b) and c) undamaged monolayer surface with intact thick carpet pattern of brush-border organization typical of cell differentiation; d) monolayer surface showing signs of freezing injury such as surface smoothing and blebbing; e) sign of cell disruption from the monolayer; f) and g) signs of monolayer shrinkage even though the surface is undamaged.

As seen in Figure 6A, when cells were frozen with or without alginate entrapment, although high recovery was found immediately post-thawing ($88\pm10\%$ and $96\pm5\%$, respectively), a decrease in cell metabolic activity was observed until 3 days post-thawing, resulting in $55\pm7\%$ and $70\pm10\%$ metabolic activity, respectively; full recovery of cell metabolic activity could only be obtained 10 days after thawing.

SEM observations revealed different responses to freezing in different regions of the monolayer (Figure 6B): both regions of undamaged cell surface, covered with typical thick carpet pattern of intact microvilli (Figures 6Ba, 6Bb and 6Bc), as well as areas with damaged cells at the monolayer's surface (Figures 6Bd and 6Be) were found within the same well. It was further observed that, although surface morphology with intact microvilli and close junctions were maintained in some areas, the typical "tissue-like" structure which grew above the average height of the monolayer suffered considerable shrinkage (Figures 6Bf and 6Bg), throughout the whole monolayer independently of the position of the cells within the well.

The effect of the cryopreservation process on the survival of N2a cells fully differentiated into neuronal networks was evaluated through phase-contrast and fluorescence microscopy after immunocytochemistry with neuronal markers and measurement of metabolic activity (Figure 7).

(A)**(B)**

(c)

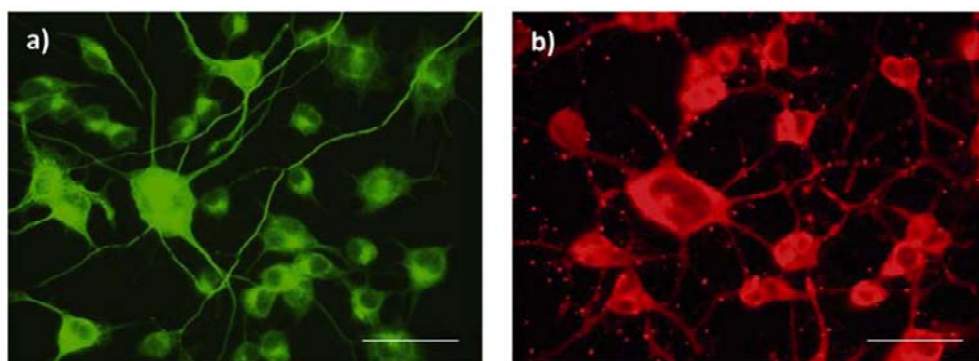


Figure 7. Effect of alginate entrapment on the recovery (A), network integrity (B) and differentiated state (C) of neuronal differentiated N2a cells after cryopreservation in CryoStor™-CS10. Cells were cryopreserved as neuronal networks without alginate or beneath a layer of alginate added 1 or 4 days post-inoculation. A: the data represents alamarBlue fluorescence immediately (black bars), 1 (spotted bars), 2 (diagonal hatched bars) and 3 (horizontal hatched bars) days post-thawing as a percentage of non-preserved controls (white bars); B: phase contrast photographs of N2a cells immediately (a and c) and 1 day (b and d) post-thawing, after cryopreservation without alginate (a and b) or beneath a layer of alginate gel (c and d); C: immunofluorescence photographs of N2a neuronal networks 1 day post-thawing when cryopreserved beneath a layer of alginate: a) labelling of β -tubulin (green) and b) MAP2a/b (red). Scale bars = 50 μ m.

As shown in Figure 7A for CryoStor™-CS10 cryopreserved N2a cells as fully differentiated neuronal networks, with or without alginate entrapment, a drop in cell metabolic activity was found immediately after thawing ($72 \pm 12\%$ and $24 \pm 6\%$, respectively). Although neuronal networks cryopreserved without alginate remained attached to the well's surface immediately after thawing (Figure 7Ba), it was found that 24 hours later neurite connections would break and cells would detach from the well's surface (Figure 8Bb) and eventually be lost during alamarBlue assays. For alginate entrapped cells, however, the neuronal network integrity was maintained (Figure 7Bd) and 100% cell metabolic activity was recovered within 3 days post-thawing. Furthermore, after 1 day in culture post-thawing, cells were expressing the typical neuronal differentiation markers beneath the alginate gel layer, as shown in Figure 7C.

Additionally, alginate could efficiently be dissolved by Na_2SO_4 without causing any damage to cell morphology or function, as shown through SEM (Figs.

5e and 5f and Fig. 6B) for Caco-2 and immunocytochemistry for N2a cells (Figure 7C).

4. DISCUSSION

Previous data from our lab has shown that routine cryopreservation of Caco-2 and N2a cell lines in suspension using a slow-freezing cryopreservation protocol in cell culture medium (containing 10% DMSO) yields over 90% recovery post-thawing (Malpique et al. 2007). However, when cryopreserved as confluent monolayers on well-plates, high loss of metabolic activity and membrane integrity after thawing were observed. These results are in accordance with previous reports of loss of viability and excessive cell detachment for different cell lines cryopreserved as monolayers (Wusteman et al. 1997; Pasch et al. 1999; Ebertz et al. 2004). The presence of cell-cell and cell-surface interactions has been shown to render cells more susceptible to freezing injury (Berger et al. 1996; Yang et al. 1996; Acker et al. 1999). Such interactions are likely sites for monolayer damage by the osmotic stresses and phase changes involved in the process of cryopreservation (Armitage et al. 1995; Armitage et al. 1996) and have been associated with enhanced susceptibility to intracellular ice formation (IIF) (Berger et al. 1996; Acker et al. 1998; Acker et al. 2001). Furthermore, the extended morphology of attached cells may create conditions for cryopreservation-induced damage to the cell ultra-structure (cytoskeleton, focal adhesions or gap junctions) due to mechanical forces, such as extracellular ice and differential thermal contraction between cells and substrates (Liu et al. 2005; Liu et al. 2006).

In the present study, cell entrapment beneath a layer of UHV alginate gel was investigated as a strategy to improve the cryopreservation of both cell lines as undifferentiated or fully differentiated monolayers. To develop biosynthetic conduits for cell culture and cryopreservation, the selected hydrogels must be tested for their compatibility with cells. Thus, the morphology of each cell line was

closely monitored during proliferation and differentiation assays before freezing. The results presented herein demonstrate that the addition of an alginate layer over cells cultured for 4 days on matrigel-coated surfaces does not influence cell growth or differentiation, which confirms the possibility of using this UHV alginate as a biocompatible matrix for cell entrapment (Clayton et al. 1993). Nevertheless, for both cell lines investigated, alginate addition 1 day post-inoculation inhibited metabolic activity and affected cell morphology and differentiation. This difference may be due to the fact that at day 1 post-inoculation most of the cells in culture were loosely attached, as indicated by their round morphology, while after 4 cells have already developed strong cell-cell and cell-surface contacts. Thus, we hypothesize that alginate addition 1 day post-inoculation inhibits cell attachment and spreading on the surface, which in turns limits cell migration and division under the alginate. This might also explain the inhibition of neurite extension of N2a cells entrapped in alginate, which may not be able to establish the proper connections with the ECM required for axon growth. Impaired cell growth in alginate has also been reported for other cell lines, such as olfactory ensheathing cells and Schwann cells (Novikova et al. 2006), whereas murine insulinoma (Simpson et al. 2004) and fibroblasts (Tobias et al. 2001) have been shown to rapidly proliferate in alginate beads. This contradictory data might be due to differences in the type and characteristics, namely different viscosities, of the alginate used, which will influence the diffusion rates of oxygen, nutrients and differentiation factors throughout the matrix as well as the mechanical constraints imposed to the cells within the gel (Torre et al. 2002; Simpson et al. 2004).

The results presented in this paper show that cryopreservation strategies based on UHV alginate gel entrapment led to improved recovery of metabolic activity and function of Caco-2 and N2a cell lines adherent to matrigel-coated culture well-plates. Our results demonstrate that alginate entrapment improves the recovery of cells cryopreserved in cell culture medium by reducing membrane damage and cell detachment immediately after thawing. Furthermore, it was

found that the proliferation and differentiation capacities during long-term culture of Caco-2 cells cryopreserved beneath an alginate layer added 4 days post-inoculation were not affected, which is a critical issue for cells intended to be used on therapeutic or pharmacological purposes immediately after thawing. Despite matrigel has previously been shown to promote improved adherent stem cells recovery after cryopreservation (Ji et al. 2004), in the present study matrigel-coating by itself had no significant impact on the survival of both cell lines, as similar recovery values were found for cells cryopreserved on uncoated and matrigel-coated surfaces (results not shown).

Cell entrapment by micro-encapsulation within alginate beads has previously been reported as a promising strategy to improve post-thaw viability and function of cell types which were shown to poorly survive the cryopreservation process, such as hepatocytes (Guyomard et al. 1996; Rialland et al. 2000; Mahler et al. 2003) and pancreatic islets (Inaba et al. 1996; Zhou et al. 1997; Yoshifumi et al. 2001; Zimmermann et al. 2007), although the underlying mechanisms are still unclear.

To our knowledge, this is the first report on the cryopreservation of adherent cells beneath a layer of UHV alginate gel. Our results suggest that monolayer entrapment beneath a layer of alginate improves cell recovery by providing cell immobilization thus avoiding detachment from the surface and breakage of cell-cell interactions. Furthermore, as a considerable increase in the number of attached cells with undamaged membranes was found immediately after thawing, it seems plausible that the presence of the gel not only avoids physical cell rupture but also mitigates cryopreservation-induced cell death. Such effect may be related with the influence of the polymeric matrix of the gel on extracellular ice formation (Murase et al. 1997; Murase et al. 2004), which is known to be involved in cryo-induced injury due to modification of the mechanical chemical and environment of the cell (Mazur et al. 1972; Tondorf et al. 1987; Karlsson et al. 1996) as well as direct involvement in initiation of ice formation

inside cells (Mazur 1965; Mazur et al. 1972; Toner et al. 1993).

Despite the improved cell recovery immediately after thawing for culture medium cryopreserved cells beneath alginate, up to 50% cell death still occurred within 24 hours post-thawing. In agreement with reports on the literature describing post-thaw survival overestimates, such decrease in cell viability might be related to sequential apoptotic and necrotic processes not evident immediately subsequent to thawing (Frim et al. 1978; Baust et al. 2000). In this sense, the use of CryoStorTM solution resulted in a considerable improvement of the cryopreservation process for both cells lines in an undifferentiated state, either with or without alginate gel entrapment: high post-thaw membrane integrity and metabolic activity as well as the structural integrity of the confluent monolayers were maintained, not only immediately after thawing but also for the following 3 days in culture.

CryoStorTM has been developed from the platform hypothermic storage solution HypothermosolTM, which is part of a number of preservation solutions currently available in the field of regenerative medicine (Taylor et al. 1995; Baust et al. 1998). These solutions have been carefully formulated to maintain the ionic and hydraulic balances of cells at low temperatures thus facilitating the preservation of cell homeostasis and control of the ionic environment that cannot be achieved using traditional preservation media formulations consisting of a basal culture media with serum protein and Me₂SO supplementation (Taylor 1982; Taylor et al. 1985; Southard et al. 1990; Taylor et al. 2001). The vehicle solutions Euro-Collins and Unisol-CPA have shown to allow the cryopreservation of two vascular cell lines as monolayers, although only 50% recovery of metabolic function immediately after thawing was obtained and full recovery could only be observed 3 days post-thawing (Campbell et al. 2007). Besides its “intracellular-type” (low Na⁺, high K⁺) composition, CryoStorTM is supplemented with several additional components to reduce the generation of free radicals or energy deprivation at non-physiological temperature and inhibit apoptosis (Baust et al.

2000). The improved cell survival and function of different cell types, such as hepatocytes, cord blood or stem cells, when cryopreserved in suspension in CryoStor™ solution, has been related to a direct reduction in the level of both apoptosis and necrosis by inhibition of cellular stress (ionic, osmotic and/or biochemical) during cryopreservation (Baust et al. 2000; Baust et al. 2001; Baust et al. 2002). We can hypothesise that a decrease in post-thaw necrotic and/or apoptotic cell death pathways is related with the improved post-thaw recovery found for the two cell lines used in the present study. However, this is the first report on the use of on the use CryoStor™ for the preservation of cell monolayers and further studies must be performed to understand the cryoprotective mechanisms.

The cryopreservation of differentiated monolayers, proved to be more difficult than for undifferentiated cells, due to the development of cell specific features such as multiple cell layers for Caco-2 or neuronal networks with formation of extensive neuritic processes for N2a cells. Cryopreservation of fully differentiated Caco-2 monolayers resulted in reduced recovery 1 day post-thawing when comparing with cells cryopreserved as undifferentiated monolayers. This might be due to additional factors influencing the cryopreservation outcome when freezing such multiple cell layer structures, which are related to the three dimensional arrangement and limitations in heat and mass transfer (Pegg et al. 1979). Full recovery of cell metabolic activity could only be obtained after 10 days in culture, which may not be acceptable for the desired applications of the monolayers on clinical and experimental settings. Thus, to achieve the desired cell recoveries, adjusting other cryopreservation parameters, such as freezing and thawing rates (Armitage et al. 1996; Karlsson et al. 1996; Pegg 2002), CPA concentration or incubation time with the CPA (Karlsson et al. 1996), may be necessary.

For the cryopreservation of differentiated N2a cells, even though cell damage was reduced when CryoStor™ was used, entrapment beneath a layer of

alginate was crucial for maintenance of attachment and integrity of the neuronal network. Ma et al. have reported on 60% cell recovery immediately post-thawing for adherent neuronal networks cryopreserved beneath a thin film of collagen gel using culture medium supplemented with high concentrations of bovine serum and DMSO and trehalose as the CPAs (Ma et al. 2004). However, no further studies were performed to assess cell recovery within the following hours post-thawing, which is of major importance for the evaluation of the full extent of post-thaw cell death due to the time course of apoptotic and necrotic mechanisms has previously reported (Frim et al. 1978; Baust et al. 2000; Baust et al. 2002). Our results show that combining the use of serum-free CryoStorTM solution and UHV alginate entrapment improves the cryopreservation of neuronal networks by maintaining proper neuronal connections and functionality and allowing 100% recovery of cell metabolic activity within 3 days post-thawing.

5. CONCLUSIONS

The combination of different strategies based on cell entrapment in UHV alginate and serum-free CryoStorTM solution lead to optimized protocols for the cryopreservation of adherent cells in a fully differentiated state. Such protocols enabled the retention of cell membrane and whole monolayer integrity, metabolic activity and cell specific function/differentiated state, a major requirement for clinical and pharmacological applications of such cell lines. Successful cryopreservation of large numbers of viable and functional adherent cells on micro-plates eliminates the time consuming process of inoculation and expansion from a frozen vial of cells, thus reducing time between cell storage and use in experimental or clinical settings. Furthermore, such protocols as developed here facilitate the creation of cellular clone banks, reducing batch-to-batch variability and normalizing passage age, which are obstacles for achieving reliable results in pharmacological testing. Finally, these protocols should support the implementation of routine cryopreservation practices during preparation of engineered cells and tissues for clinical applications and enable the immediate availability of cells grown on biocompatible matrices for transplantation to treat several injuries (e.g. spinal cord, cartilage, skin, cornea, etc).

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CRYOPRESERVATION OF 3-D AGGREGATES

Alginate Encapsulation as a Novel Strategy for the Cryopreservation of Neurospheres

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ABSTRACT

Primary cultures of brain cell neurospheres are valuable *in vitro* models for neurotoxicology and brain cell research. Such applications would greatly benefit from the development of efficient cryopreservation protocols that assure the availability of viable and genetically stable stocks of functional neurospheres. In this work we aimed at developing an integrated strategy allowing for long-term culture and cryopreservation of brain cell neurospheres with high viability and reduced recovery time post-thawing.

Micro-encapsulation in clinical-grade, ultra-high viscous, highly purified alginate uniformly cross-linked with Ba^{2+} was evaluated as the main strategy to avoid the commonly observed loss of cell-cell and cell-matrix interactions with consequent aggregate's fragmentation and decrease in cell viability that occurs post-thawing.

Brain cells isolated from 16-day old fetal rats were cultured in spinner vessels as neurospheres, encapsulated at the 5th day of culture and cryopreserved at day 19. Culture characterization and assessment of post-thawing recovery, concerning cell metabolism, aggregate's cell type composition and neurons-astrocytes interactions, were performed through analysis of membrane integrity, metabolic activity assays and immunohistochemistry.

Our results show that the encapsulation process does not affect cell viability central metabolism, neither cell differentiation nor the cell extensions into cell networks usually observed between neurons-astrocytes within the neurospheres structure. Neurospheres encapsulation resulted in reduced recovery time post-thawing and significantly less fragmentation. Furthermore, the use of serum-free CryoStorTM solution provided further protection for both non-encapsulated and encapsulated aggregates when compared to serum-supplemented culture medium as the cryopreservation medium.

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1. INTRODUCTION

Primary cultures of brain cell aggregates, commonly known as brain spheroids or neurospheres, can be obtained from mechanically dissociated embryonic brain tissue that self-aggregate to form even-sized, spherical structures. Such cultures have many advantages over monolayer cultures related with their well-organized three-dimensional (3-D) spatial arrangement constituted by different cell types (including neurons, astrocytes, oligodendrocytes and microglia) (Seeds 1971; Santos et al. 2007). These cells are able to perform specific cellular interactions through direct cell-cell contact and exchange of nutritional and signaling factors as well as to develop specialized structures resembling those of brain tissue *in situ*, including patterns of morphological differentiation (Seeds et al. 1971; Honegger et al. 1977), spontaneous electrical activity (Stafstrom et al. 1980), and synthesis, storage and release of neurotransmitters (Honegger et al. 1979; Santos et al. 2007). Thus, primary brain neurospheres are well established as valuable *in vitro* model systems for neurotoxicology (Honegger et al. 1988), brain research (Matthieu et al. 1979; Wehner et al. 1985) and tissue engineering (Mueller-Klieser 1997). Moreover, our group reported recently on the use of stirred tank vessels and bioreactors for the maintenance of 3-D primary cultures of different cells, namely brain cells, stem cells and hepatocytes (Santos et al. 2007; Serra et al. 2007; Miranda et al. 2009). This culture system allows for on-line monitoring and control of critical cell culture parameters such as oxygen content, pH and as neurosphere size (avoiding necrotic centers). Furthermore, non-invasively sampling and cell harvesting over long time culturing periods is possible.

Neurospheres applications in research and therapy would greatly benefit from the development of efficient cryopreservation strategies that assure the availability of viable and functional stocks of neurospheres, thus reducing batch-to-batch variation and eliminating the laborious, time-consuming process of cell isolation from the animal.

Cryopreservation at temperatures below -130°C using 5% to 20% dimethyl sulfoxide (DMSO) in serum-supplemented culture medium and slow-freezing-rates is a routine procedure for the prolonged storage of many mammalian cell types, which works reasonably well for suspended single-cells. However, for 3-D multicellular systems (e.g. neurospheres) the different permeability and cryotolerance of the various cell types combined with heat and mass (water and cryoprotectants (CPAs)) diffusion restrictions leads to differences in the freezing environment experienced by individual cells (Mazur et al. 1972; Karlsson et al. 1996; Ehrhart et al. 2009). This results in the loss of cell-specific function and/or metabolic activity, reduced cell number in comparison to unfrozen controls, disintegration and shape distortion (Merchant et al. 1993; Purcell et al. 2003; Ehrhart et al. 2009).

Alternatives to the standard cryopreservation protocols have been proposed, namely entrapment on extracellular matrices or gels (Koebe et al. 1990; Ji et al. 2004; Malpique et al. 2009), the use of alternative CPAs to DMSO (Pasch et al. 1999; Ehrhart et al. 2009) or the use of solutions specially designed to reduce the ionic and hydraulic imbalances induced in cells during hypothermia (Taylor et al. 1985; Malpique et al. 2009). Encapsulation is a promising approach for cryopreservation of 3-D cell-systems as cells are protected against mechanical damages during ice crystallisation and the danger of disrupting cell-cell contacts are reduced through immobilization within the hydrogel (Inaba et al. 1996; Zimmermann et al. 2007). Furthermore, gel entrapment brings advantages to cell neurospheres in culture as it provides protection from hydrodynamic shear and prevention of fragmentation while allowing diffusion of nutrients and proteins due to the pore size (Zimmermann et al. 2007). Amongst the different polymers used in biotechnological and medical applications, alginates are very versatile protein-free, well characterized immuno-protecting, ionotropic matrices for immobilization of single cells (Glicklis et al. 2000) and multicellular aggregates (e.g. Langerhans' islets and parathyroid tissue) (Clayton et al. 1993; Hasse et al.

1997; Schneider et al. 2005). Clinical-grade, highly purified alginate of extremely high viscosity (0.1% w/v viscosity in distilled water > 30 mPa.s) extracted by a novel process and uniformly cross-linked with Ba^{2+} is an effective matrix for the entrapment of cells, tissues or other biologically active material (Zimmermann et al. 2005). Its ability to improve cell viability after thawing of differentiated cell monolayers cryopreserved beneath an alginate layer has been recently described by our group (Malpique et al. 2009).

In this work, micro-encapsulation in ultra-high viscous (UHV) alginate was evaluated as the main strategy for avoiding the commonly observed neurospheres fragmentation and loss of cell-cell/matrix interactions post-thawing. Optimization of critical process parameters, such as alginate concentration and polymerization time with BaCl_2 , was done using the neuroblastoma N2a cell line as a model system due to its capacity to differentiate into randomly connected neuronal populations (Thiele 1998). Additionally, to further potentiate the advantages of alginate encapsulation, standard cell culture media was compared with protein- and serum-free CryoStorTM solution as the vehicle solution for the CPA (DMSO). CryoStorTM is a cryo-medium designed to meet the specific, molecular requirements of individual cells and tissues, which has been reported to significantly enhance post-thaw cell survival and recovery in a series of cellular systems (Elford et al. 1972; Baust et al. 2001; Sosef et al. 2005; Stylianou et al. 2006; Malpique et al. 2009).

2. MATERIALS AND METHODS

2.1. MATERIALS

Basal medium Eagle's (BME), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine, penicillin-streptomycin, phosphate-buffered saline (PBS), trypsin-EDTA (1x solution). DMSO, fluoresceine diacetate and ethidium bromide were obtained from Sigma (Steinheim, Germany).

AlamarBlue was obtained from Invitrogen (Paisley, UK). Culture plastic-ware was obtained from Nunc (Roskilde, Denmark). CryoStorTM-CS10 was obtained from BioLife Solutions (Bothell, WA, USA). The monoclonal antibodies used were specific to glial fibrillary acidic protein (GFAP; Boehringer Mannheim GmbH, Mannheim, Germany) and to neuron-specific β III-tubulin (Chemicon, Hampshire, United Kingdom). Other chemicals were of the purest grade available from regular commercial sources.

Studies on stirred vessels were performed in glass 125 or 250 ml spinner flasks (Wheaton, Techne, NJ). The spinners were siliconized prior to experiments to avoid cellular adhesion to the inner surface of the spinner and the outer surface of the ball impeller.

Alginates: Clinical grade, UHV alginates were produced and purified from stipes of *L. nigrescens* and *L. trabeculata* as described elsewhere (Zimmermann et al. 2001). A 1:1 mixture of purified UHV alginates from those two species was used at 0.4% or 0.7% (w/v) in 0.9% NaCl solution.

2.2. N2A CELLS CULTURE

The N2a cell line was obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air DMEM supplemented with 10% (v/v) heat inactivated FBS and 4.5g/l glucose and sub-cultured every 5 days. All experiments were performed using N2a cells between passages 10 to 30.

2.3. PRIMARY CULTURES OF BRAIN NEUROSPHERES

2.3.1. Culture Preparation and Spinner Inoculation

Primary cultures of brain neurospheres were prepared with cerebral hemispheres removed from 15-16-day-old Wistar rat embryos (Harlan Iberica, Barcelona, Spain). The dissociation procedure was adapted from Yavin and Yavin

(1974) and has previously been described (Santos et al. 2007). Single-cell suspensions (approximately 2×10^8 cell/spinner flask) were seeded in 2 250 ml spinner flasks with ball impeller containing 80-100 ml of the supplemented medium, at 60 rpm, and kept at 37°C in a humidified atmosphere of 7% CO₂ in air. After 2 hr of culture, the volume was increased to 160-200 ml, and the agitation rate was increased to 80 rpm. After 24 hr, culture medium was added for a final culture volume of 250 ml.

2.3.2. Culture Maintenance

Primary cultures of brain neurospheres were maintained in spinner flasks for 19 days until cryopreservation was performed. For maintenance of the neurospheres, a refeed operational mode with 50% medium substitution approximately twice per week was applied. The replacement criterion was to maintain glucose concentration above 2mM. Medium substitutions were performed in a laminar flow cabinet with agitation stopped and gentle removal/refeeding of medium after sedimentation of neurospheres (Santos et al. 2007).

2.4. CELL ENCAPSULATION IN UHV ALGINATE

N2a cells: Cells were harvested from monolayer cultures using trypsin-EDTA and suspended in UHV alginate at a density of 1×10^6 cell/ml alginate.

Neurospheres: Neurospheres from 1 of the spinner vessel were encapsulated after 5 days of culture by re-suspending the pellet in 3 ml of UHV alginate.

2.4.1. Micro-Capsules Formation

Micro-capsules formation was achieved by using either the conventional air-jet two-channel droplet generator or the “crystal-gun” method (Figure 1) as described in detail elsewhere (Zimmermann et al. 2001), yielding capsules with a

diameter of approximately 400 μm . For cross-linkage of the UHV alginate, capsules were dropped directly from the droplet generator's nozzle into a 20 mM BaCl_2 solution, adjusted to 290 mOsm using NaCl buffered at pH 7 with 5 mM histidine.

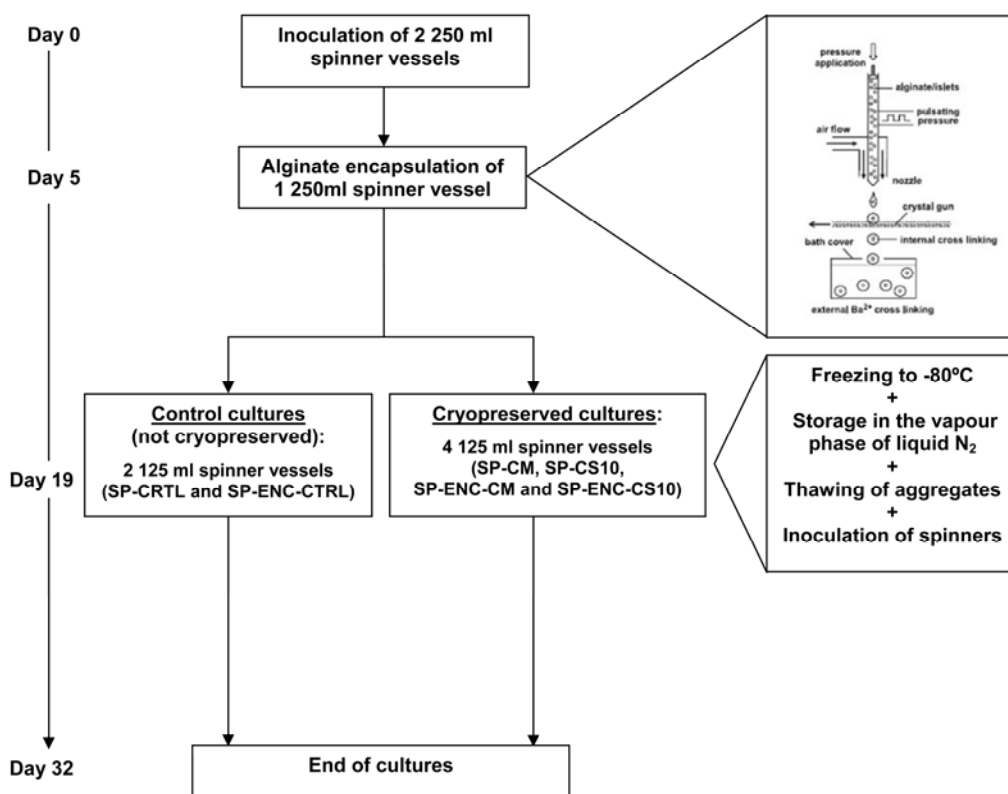


Figure 1. Schematic workflow of the encapsulation, cryopreservation and post-thawing evaluation of neurospheres cultures. Details of the air jet droplet generator equipped with a “crystal gun” for internal and external gelling of alginate micro-capsules (adapted from (Zimmermann et al. 2007)) and the steps of the cryopreservation process and the post-thawing recovery analysis.

N2a cells: After 2 or 20 min incubation the micro-capsules were washed three times with 0.9% NaCl solution and the encapsulated single-cells were cultured in petri-dishes. Non-encapsulated controls were cultured on static culture flasks.

Neurospheres: UHV alginate micro-capsules were polymerized for 20 min, washed three times with 0.9% NaCl solution and cultured in a 250 spinner vessel (Sp-Enc), respectively. Non-encapsulated controls (Sp) were cultured under the same conditions.

2.5. CELL CRYOPRESERVATION

Encapsulated and non-encapsulated N2a cells were harvested from the petri-dishes after 1 or 7 days of culture, centrifuged (200 *g*, 5 min), re-suspended in serum-supplemented culture medium and transferred to cryovials (1 ml/vial).

Encapsulated and non-encapsulated neurospheres were harvested from the spinner flasks after 19 days of culture and centrifuged (200 *g*, 5 min). The pellet from each spinner was divided in 3 parts and re-suspended in culture medium for inoculation of two 125 ml spinners (unfrozen controls: Sp-CRT and Sp-Enc-CRT) or in the cryopreservation medium and transferred to the cryovials (1 ml/vial). The cryopreservation medium consisted of either serum-supplemented culture medium (CM) or serum-free CryoStorTM-CS10 solution (CS10), both containing 10% DMSO. The cells were allowed to equilibrate in the cryopreservation medium for 30 min at 4°C. Samples were cooled at 1°C/min to -80°C in a rate-controlled freezing system (Planer Kryo 560-16, Planer, Middlesex, UK) and stored in the vapour phase of liquid N₂ for 1-2 weeks.

Following storage, cells were quickly thawed by placing the cryovials in a 37°C water bath, a two-step dilution (1:2 and 1:6) in culture medium was performed immediately after thawing and the cells were cultured at 37°C and 5% CO₂ for 30 min before total growth medium replenishment. Cells were further cultured for post-thaw studies of cell viability, function and differentiation state. N2a cells were cultured in petri-dishes. Brain neurospheres were cultured in 125ml spinner vessels: Sp-CM and Sp-Enc-CM for culture medium cryopreserved spheroids; Sp-CS10 and Sp-Enc-CS10 for CryoStorTM-CS10 cryopreserved spheroids.

2.6. CULTURE CHARACTERIZATION AND POST-THAW RECOVERY ASSAYS

N2a cells: Cell membrane integrity was monitored on a daily basis during at least 3 days post-thawing.

Neurospheres: Samples of the culture supernatant were taken on a daily basis during the whole culture time (prior to freezing and after thawing) for analysis of cellular viability and metabolic activity. For alamarBlue assays, evaluation of cell membrane integrity and biomass quantification, samples were collected twice a week before freezing, on a daily basis during the first three days post-thawing and thereafter twice a week. Cell growth and recovery after thawing was also monitored during 3 days by visualization on an inverted microscope (Leica DM IRB). Whenever required, Ba²⁺ cross-linked alginate was dissolved by incubating with a 20 mM Na₂SO₄ solution for 20 min at 37°C in a humidified atmosphere of 5% CO₂ in air.

2.7. ANALYTICAL METHODS

2.7.1. Evaluation of Cell Membrane Integrity

The qualitative assessment of the cell plasma membrane integrity during culture was done using the enzyme substrate fluorescein diacetate (FDA) and the DNA-dye ethidium bromide (EB) as described in the literature (Malpique et al. 2007).

2.7.2. Evaluation of the Culture Metabolic Status

2.7.2.1. Quantification of Metabolites in the Supernatant

Glucose, glutamine, and lactate concentrations were analyzed by using an YSI 7100 Multiparameter Bioanalytical System (Dayton, OH). Ammonia was quantified enzymatically via UV assay (Boehringer Mannheim, R-Biopharm AG, Darmstadt, Germany).

2.7.2.2. AlamarBlue Assay

Metabolic activity was assessed using the non-toxic metabolic indicator, alamarBlue following the manufacture's recommendation. Briefly, a 4ml sample from the spheroids cultures were placed in 25 ml siliconized glass flasks, incubated with fresh medium containing 10% alamarBlue and 7 hours later fluorescence was measured in 96-well plates using a microplate fluorescence reader (Biotek Instruments, Winooski, VT, USA).

2.7.3. Evaluation of Neurosphere Cellular Composition

Immunocytochemical analysis in the whole neurosphere was performed before and after cryopreservation. The monoclonal antibodies used were specific to glial fibrillary acidic protein (GFAP) and to neuron-specific β III-tubulin. For fixation of the neurospheres, these were incubated in 4% (v/v) paraformaldehyde solution for 1 hr at 4°C and washed with PBS. The immunocytochemistry protocol was then performed as described in literature (Serra et al. 2007), except for the washing, blocking and permeabilization solutions used (0.08% (v/v) Tween 20 in PBS and 2% (w/v) BSA and 1% (v/v) Triton-X 100 in PBS, respectively) and the duration of the blocking/permeabilization step, which consisted in 2 hr of incubation. The neurospheres were mounted on glass slides and covered with a glass coverslip.

2.7.4. Transmission Electron Microscopy (TEM)

To evaluate the structural integrity and differentiation state of N2a neurospheres inside UHV alginate micro-capsules, TEM was performed after 7 days of culture prior to freezing and immediately after thawing. Encapsulated neurospheres were prepared as described elsewhere (Katsen et al. 1998) and TEM studies were performed on field emission scanning electron microscope type FESEM XL30 (Philips, Eindhoven, The Netherlands).

2.8. DATA ANALYSIS AND STATISTICS

All data presented shows $n \geq 6$ experimental replicates (minimum of 2 experimental repeats with 3 sample replicates per experiment). Error bars denote the standard deviation of the mean. Fluorescence units were converted to percent recovery based upon non-cryopreserved experimental controls (mean \pm SEM). For membrane integrity, immunocytochemistry and SEM assays representative photographs are depicted.

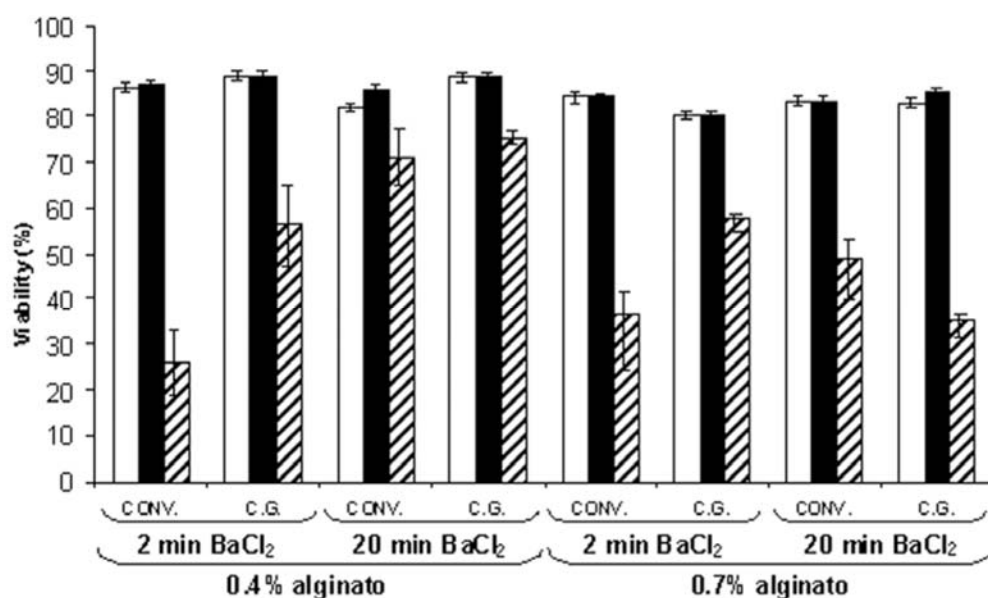
Statistical difference between cryopreservation conditions was assessed using single/multi-factor analysis of variance (ANOVA). The least significant difference (LSD) method was chosen for multiple contrasts. A 95 % confidence level was considered to be statistically significant.

3. RESULTS

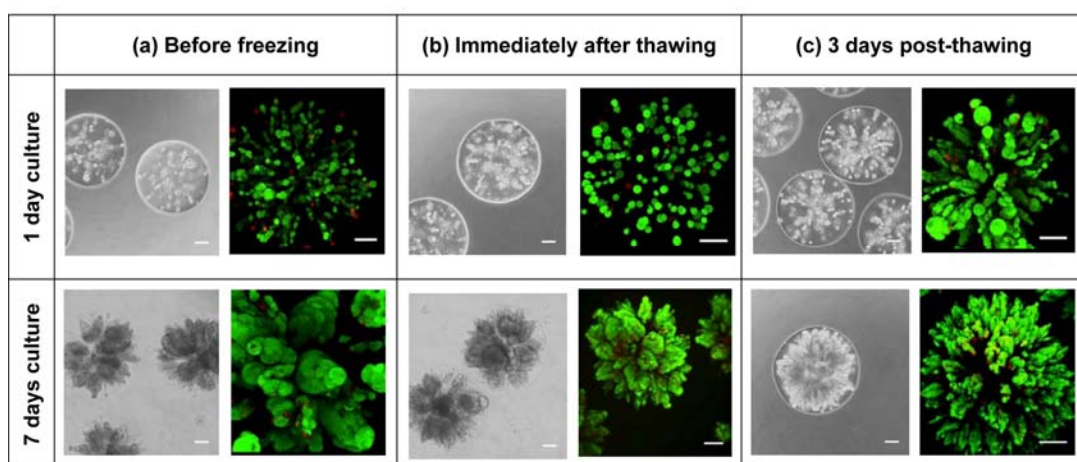
3.1. OPTIMIZATION OF ENCAPSULATION PARAMETERS

To investigate the impact of culturing and cryopreserving brain neurospheres inside UHV alginate micro-capsules, optimization of critical encapsulation parameters, namely alginate concentration, polymerization time and encapsulation method (conventional *versus* “crystal gun”), was performed by evaluating their effect on the post-thaw viability of N2a cells cryopreserved after 1 or 7 days of culture post-encapsulation (Figure 2).

(A)



(B)



(C)

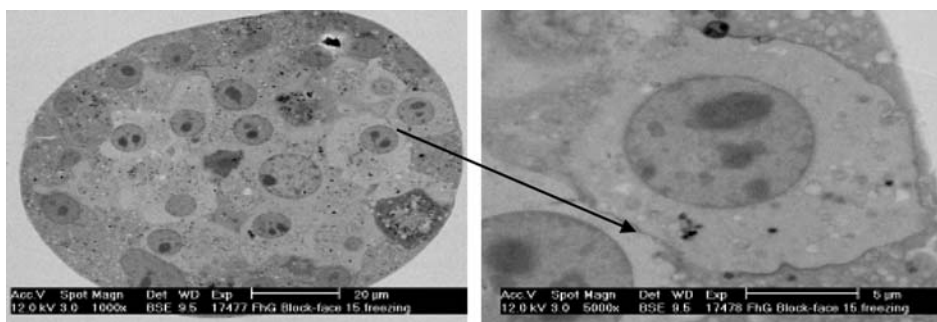


Figure 2. Effect of encapsulation parameters on the cryopreservation of encapsulated N2a cells - alginate concentration, incubation time with BaCl_2 and encapsulation method (conventional (CONV.) *versus* “crystal-gun” (C.G.) method). **A:** Membrane integrity of cells cryopreserved after 1 day of culture post-encapsulation. The data represents the number of cells with intact membranes as a percentage of the total number of cells recovered after encapsulation (white bars), before freezing (black bars) and immediately after thawing (diagonal hatched bars); **B:** Membrane integrity before freezing (a), immediately (b) and 3 days (c) post-thawing of cells cryopreserved at day 1 or 7 of culture post-encapsulation. Green cells with undamaged membranes and metabolic activity. Scale bars = 50 μm ; **C:** SEM photographs of cells cryopreserved at day 7 of culture post-encapsulation and analyzed immediately after thawing, revealing intact plasma membrane integrity and close connections between cells. **(B)** and **(C)** refer to aggregates encapsulated in 0.4% alginate and polymerized by 20 min incubation with BaCl_2 .

As shown in Figure 2A, for micro-capsules polymerized by 20 min incubation with BaCl_2 , 0.4% alginate higher cell recover was obtained (approx. 80%) after thawing when compared to the high concentrated 0.7% alginate (approx. 50% cell recovery). For micro-capsules polymerized by 2 min incubation with BaCl_2 , either with 0.4% or 0.7% alginate, the recovery of encapsulated cells was much lower (<30%). Membrane integrity tests performed at the 3rd day of culture post-thawing confirmed the results obtained immediately after thawing as shown in Figure 2B. Cells encapsulated in 0.4% alginate beads and polymerized for 20 min with BaCl_2 were able to recover from the cryopreservation process and start proliferating. This was valid for cells cryopreserved either 1 or 7 days post-encapsulation, which corresponded to single cells or closely-packed cell aggregates inside the micro-capsules. SEM analysis of the thawed cell aggregates inside the micro-capsules confirmed the maintenance of cell membrane integrity and close cell-cell junctions within each aggregate (Figure 2C).

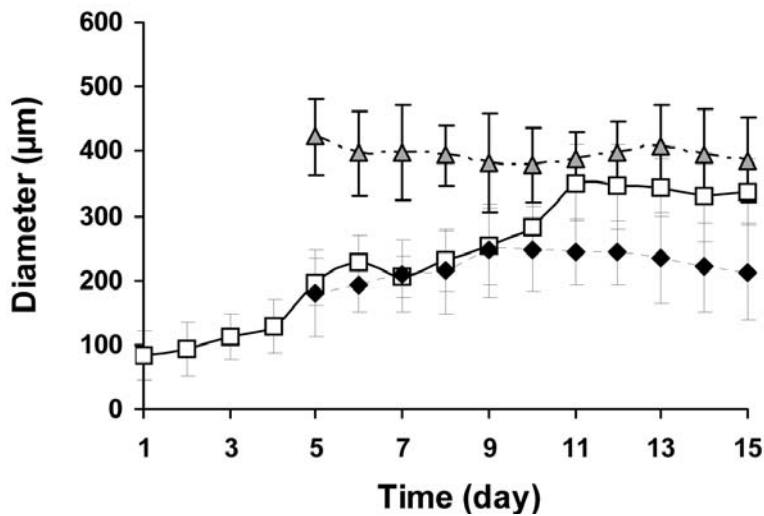
The best conditions found for the cryopreservation of both single-cells and cell aggregates, i.e., 0.4% UHV alginate polymerized by 20 min incubation with BaCl_2 , were chosen for the encapsulation studies with primary cultures of rat neurospheres.

3.2. PRIMARY CULTURES OF BRAIN NEUROSHERES

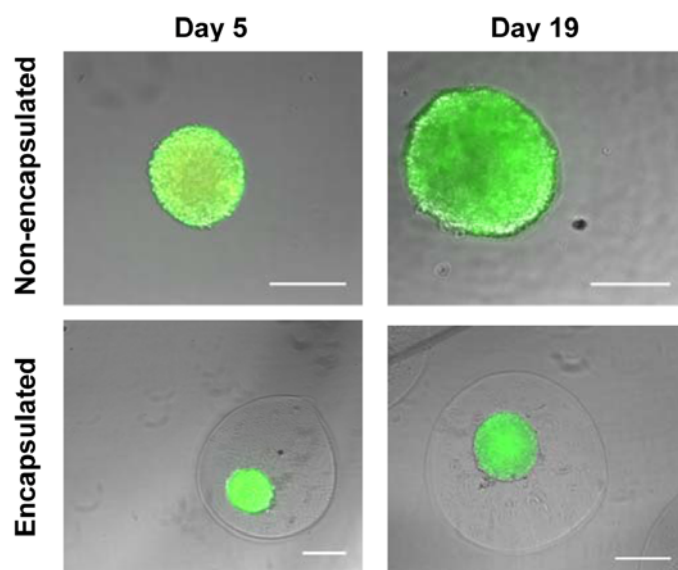
3.2.1. Effect of UHV Alginate Encapsulation on Neurosphere Size, Shape and Viability

The stability of the micro-capsules and neurospheres was monitored daily through phase contrast microscopy analysis of their size and morphology (Figure 3).

(A)



(B)



(C)

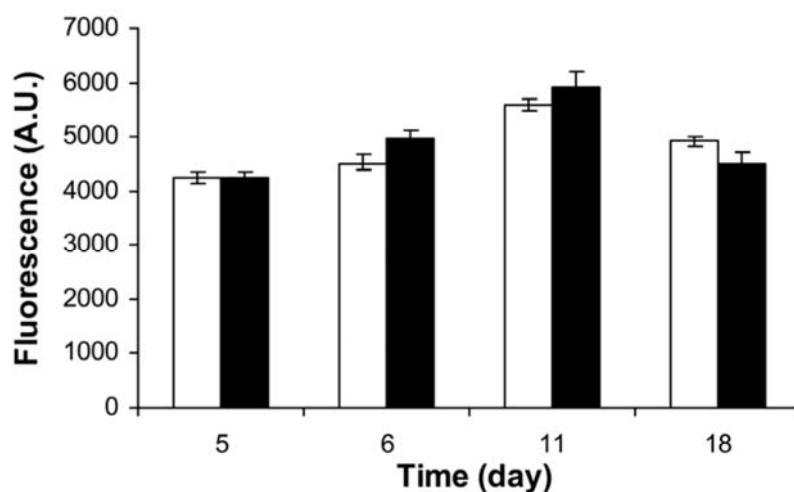


Figure 3. Effect of culture time in non-encapsulated and encapsulated neurospheres morphology and viability in spinner vessel culture. **A:** Average diameter of non-encapsulated (white squares) and encapsulated (black diamonds) neurospheres and alginate capsules (grey triangles); **B:** Membrane integrity of non-encapsulated or encapsulated neurospheres at day 5 (after encapsulation) and day 19 (before freezing). Green cells with undamaged membranes and metabolic activity; red cells with damaged membranes. Scale bars = 200 μ m; **C:** AlamarBlue viability test of non-encapsulated (white bars) and encapsulated (black bars) neurospheres culture prior to freezing.

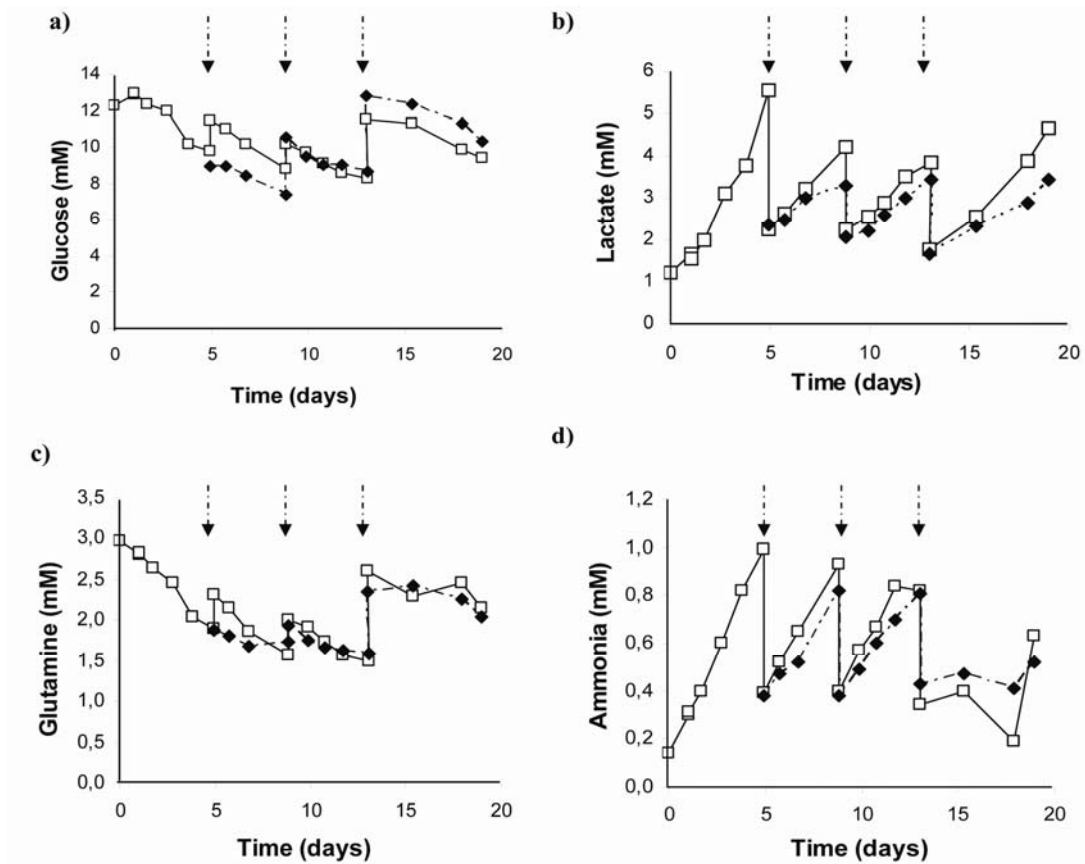
As shown in Figure 3A, the average diameter of non-encapsulated neurospheres increased from 200 μm (day 5) to 400 μm (day 19). This phenomena is mainly due to the fusion of small neurospheres (Santos et al. 2007), which, due to physical constraints, does not occur for micro-encapsulated neurospheres that show an average diameter of 200 μm up to the 19th day of culture. In this case, well-defined spherical shape was observed over culture time. It is worthwhile mentioning that UHV alginate capsules size (approximately 400 μm) and spherical shape was also maintained through culture time confirming that these matrices are robust for cell culture under stirred conditions.

Cell viability was high similar for both non-encapsulated and encapsulated neurospheres as confirmed by membrane integrity tests with a dual fluorescence assay (Figure 3B) and metabolic activity using the alamarBlue assay (Figure 3C). Overall, the results presented in Figure 3 show that the encapsulation process did not affect neurospheres morphology and cell viability.

3.2.2. Effect of UHV Alginate Encapsulation on Neurosphere Cellular Composition and Metabolism.

To assess cell population type and cell metabolic interactions within the neurospheres, glucose (Glc), lactate (Lac), glutamine and ammonia uptake and/or production were monitored (Figure 4 and Table 1).

(A)



(B)

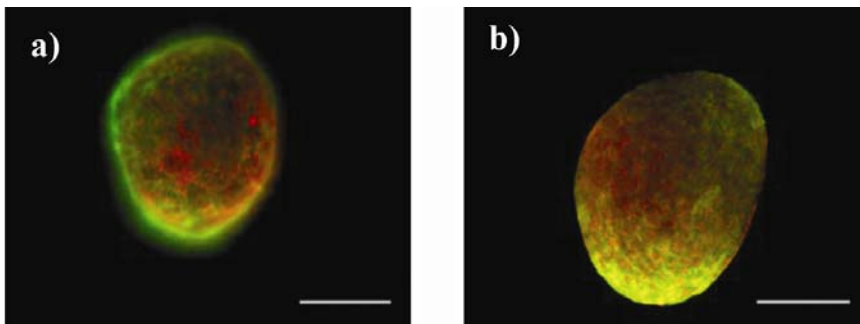


Figure 4. Cell metabolism and composition of non-encapsulated and encapsulated neurospheres. **A:** Profiles of glucose (a), lactate (b), glutamine (c) and ammonia (d) concentrations in the culture supernatant of non-encapsulated (white squares) and encapsulated (black diamonds) neurospheres cultures. Arrows represent 50% culture medium substitutions; **B:** Confocal microscopy photographs of immunofluorescence labelling of glial fibrillary acidic protein (green) and β -III Tubulin (red) in non-encapsulated (a) and encapsulated (b) neurospheres after 19 days of culture. Scale bars = 200 μ m.

Table I. Effect of alginate encapsulation on the ratios of produced lactate *per* consumed glucose in neurospheres cultures. SP / SP-ENC - non-encapsulated / encapsulated neurospheres cultures.

Time period (days)	Ratio Lac/Glc	
	SP	SP-ENC
5 - 9	0.7 ± 0.2	0.6 ± 0.3
9 - 13	0.7 ± 0.3	0.7 ± 0.3
13 - 19	1.0 ± 0.3	0.9 ± 0.3
19 - 26	0.9 ± 0.3	0.8 ± 0.2
26 - 32	1.0 ± 0.3	0.9 ± 0.3

Results presented in Figure 4A show that the encapsulation process did not affect the glycolytic metabolism of the cells as similar glucose and glutamine consumption and lactate and ammonia production profiles were obtained for both cultures. Furthermore, as shown in Table I, the Lac/Glc ratios are in agreement with previous studies performed with cell monolayers (Schousboe et al. 1997) and neurospheres (Santos et al. 2007), i.e. from days 1 to 5, when cells adapt to the culture conditions and neurospheres are formed, Lac/Glc is approximately 1.0, from days 5 to 14, essentially neuronal metabolism occurs, with Lac/Glc (0.3-0.5) similar to that of monotypic cultures of neurons, which have a lower lactate production rate than astrocytes and are able to uptake extracellular lactate; after day 14, the Lac/Glc ratio resembles neurons and astrocytes cocultures (average of 0.8) as the astrocytic population, which has a tremendous glycolytic capacity compared with neurons, starts to proliferate and mature.

Glutamine and ammonia profiles are also similar for both cultures: until the 14th day of culture, glutamine is highly consumed and ammonia highly

produced, which is typical of neuron-enriched culture behaviour; between days 14 and 19, glutamine consumption decreases and ammonia concentration increases.

Immunohistochemistry analysis using anti-glial fibrillary acidic protein (anti-GFAP) and anti- β III-tubulin antibodies (Figure 4B) show an increase in the neuronal phenotype expression from day 7 to day 14 and an effective growth of the astrocytes after day 19. These results support the outcome of metabolic studies, i.e. the encapsulation process did not affect the cellular composition of the neurospheres over time (Figure 4A) and are in agreement with previous reports in the literature obtained for non-encapsulated neurospheres (Santos et al. 2007).

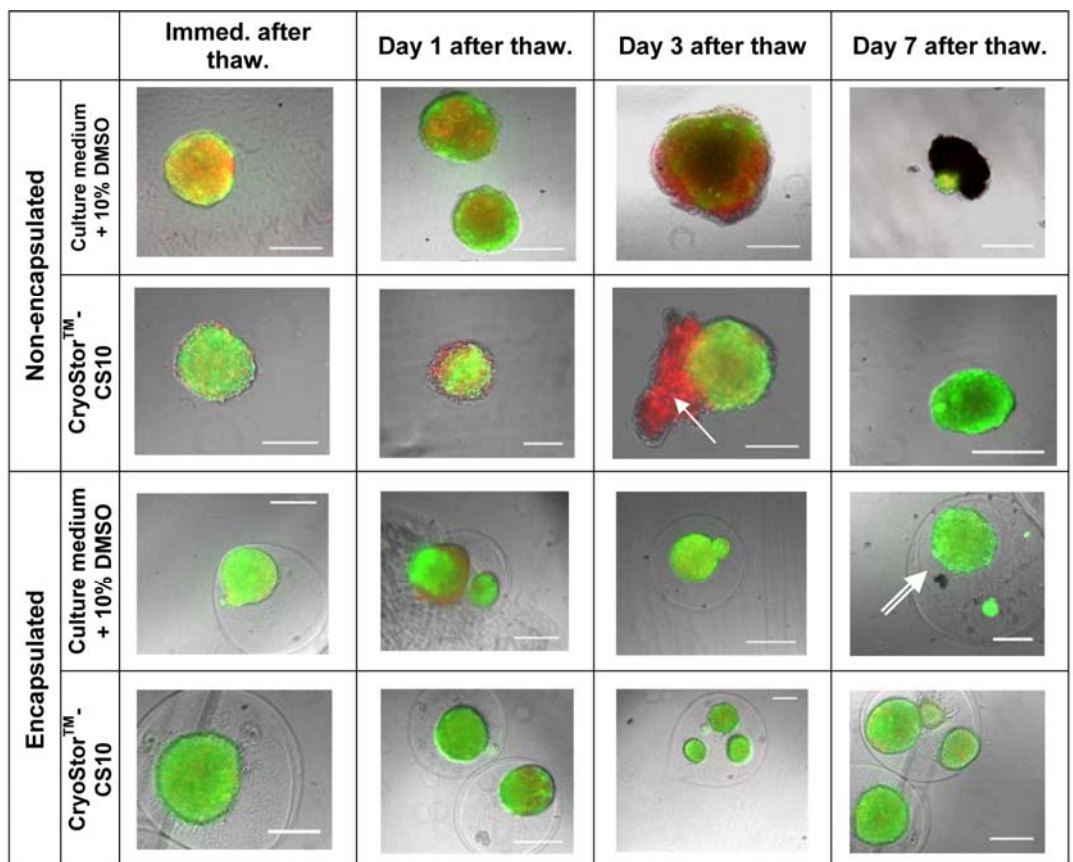
Overall, the results described above indicate that neurospheres could efficiently be cultured inside UHV alginate micro-capsules for at least 19 days.

3.3. CRYOPRESERVATION OF BRAIN NEUROSPHERES

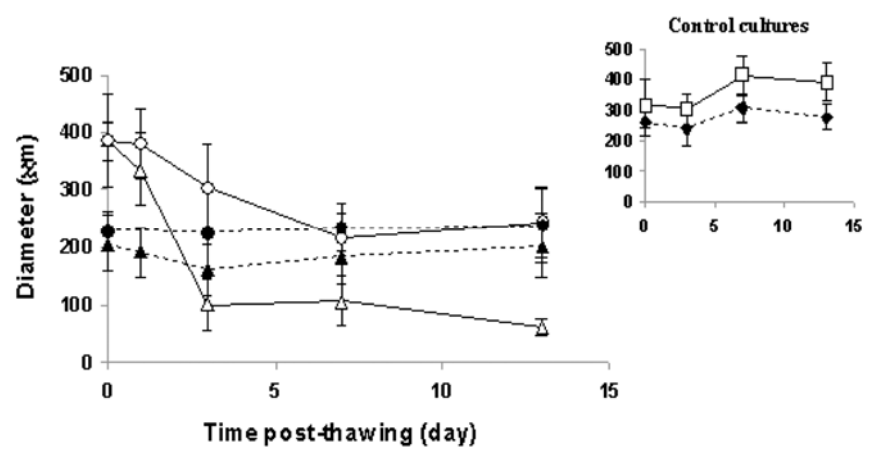
3.3.1. Effect of UHV Alginate Encapsulation and Cryomedium on Post-thaw Integrity and Viability

The effect of the cryopreservation process on neurospheres integrity and viability when returned to culture conditions post-thawing was assessed through membrane integrity tests and morphologic analysis of neurospheres disintegration and measurement of the neurospheres metabolic activity (Figure 5).

(A)



(B)



(C)

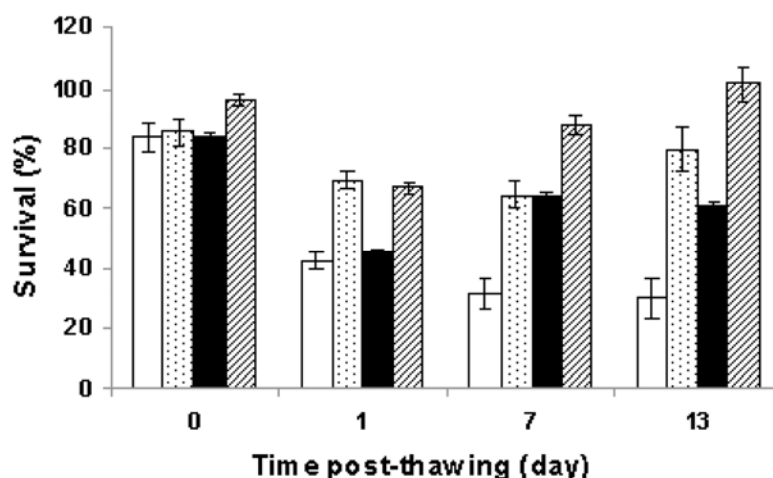


Figure 5. Post-thawing recovery of non-encapsulated and encapsulated neurospheres. **A:** Membrane integrity of non-encapsulated and encapsulated neurospheres immediately, 1 day, 3 days and 7 days after thawing. Cells with undamaged membranes and metabolic activity are labelled in green and cells with damaged membranes are labelled in red. Scale bars = 200 μ m. Single stranded arrows show dead cells disaggregating. Double stranded arrows evidence empty space inside the capsule indicating post-thawing aggregate disintegration; **B:** Average diameter of non-encapsulated aggregates cryopreserved in culture medium (white triangles) or Cryostor™-CS10 solution (white circles) and encapsulated neurospheres cryopreserved in culture medium (black triangles) or Cryostor™-CS10 solution (black non-encapsulated (white squares) and encapsulated (black diamonds) neurospheres; **C:** AlamarBlue viability test of post-thawing culture of non-encapsulated neurospheres cryopreserved in culture medium (white bars) or Cryostor™-CS10 solution (spotted bars) and encapsulated neurospheres cryopreserved in culture medium (black bars) or Cryostor™-CS10 solution (diagonal hatched bars). Survival is presented as a percentage of non-cryopreserved control cultures.

As depicted in Figures 5 , cryopreservation of non-encapsulated neurospheres resulted in loss of spherical shape, cell disintegration and a significant decrease in neurospheres average diameter for both cryopreservation solutions tested (Figure 5A - 1st and 2nd rows and Figure 5B). This decrease in diameter was due to the peel-off of dead cells (red) from the neurospheres and was less pronounced when CryoStor™-CS10 was used. It is worthwhile to highlight that non-encapsulated neurospheres cells were able to recover from the cryopreservation-induced damage 7 days after thawing when cryopreserved with CryoStor™-CS10 (Figure 5 – 2nd row, 4th column). For encapsulated neurospheres

no significant fragmentation and loss of spherical shape was observed for both cryopreservation solutions tested (Figure 5A – 3rd and 4th rows). In fact, post-thawed neurospheres average diameter was maintained when CryoStorTM-CS10 was used and only a slight decrease was found for culture medium cryopreserved samples (Figure 5B).

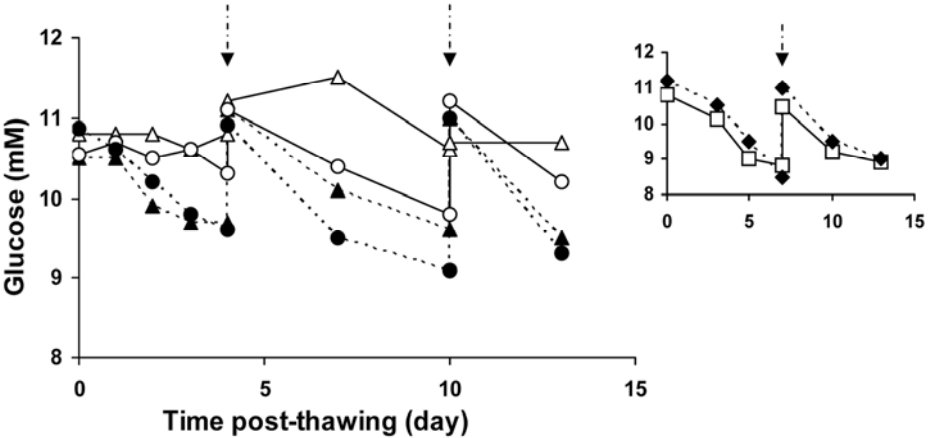
Both membrane integrity (Figure 5A) and alamarBlue metabolic activity tests (Figure 5C) show that, for all experimental conditions tested, cell survival was high immediately post-thawing and decreased within the first 24 hours of culture. However, whereas non-encapsulated neurospheres cryopreserved in culture medium could not recover and cell survival further dropped leading to an almost complete loss of cell metabolism after 7 days of culture post-thawing, non-encapsulated neurospheres cryopreserved in CryoStorTM-CS10 and encapsulated neurospheres cryopreserved in both solutions tested started to recover from the cryopreservation-induced damage within 3 days post-thawing. Nevertheless, only for encapsulated neurospheres cryopreserved in CryoStorTM-CS10, cell death and aggregate disintegration were avoided during post-thawing culture (Figure 5A).

3.3.2. Effect of UHV Alginate Encapsulation and Cryomedium on Post-thaw Cellular Composition and Metabolism

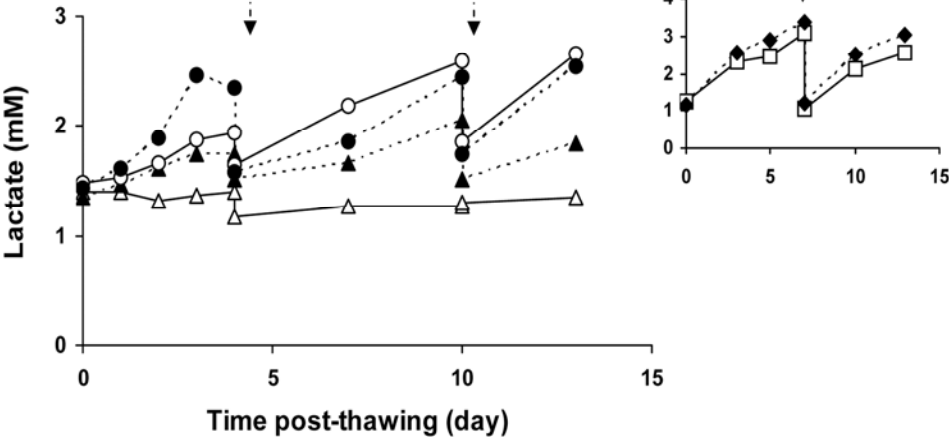
Cell population and metabolic interactions within the neurospheres were assessed through measurements of Glc, Lac, glutamine and ammonia uptake and/or production (Figure 4A and Table 1). Cellular composition was confirmed through immunocytochemistry analysis (Figure 4B).

(A)

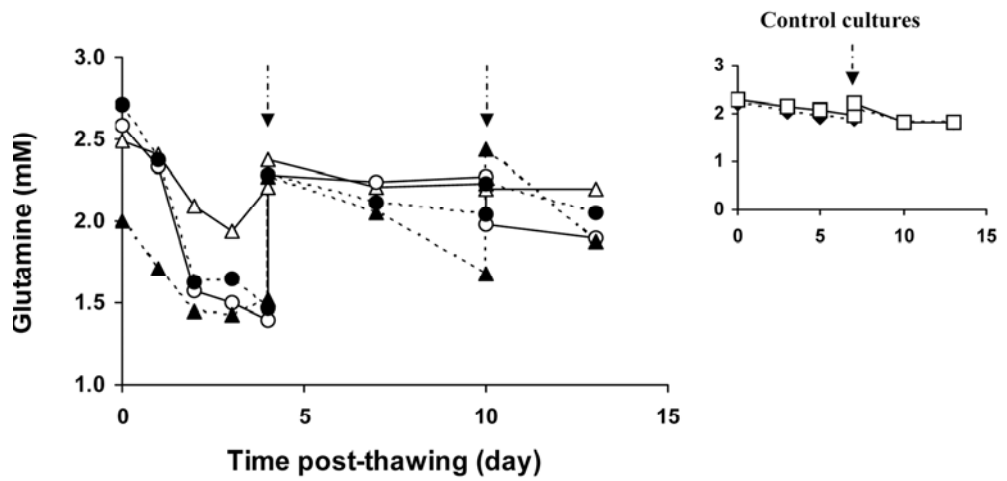
(i)



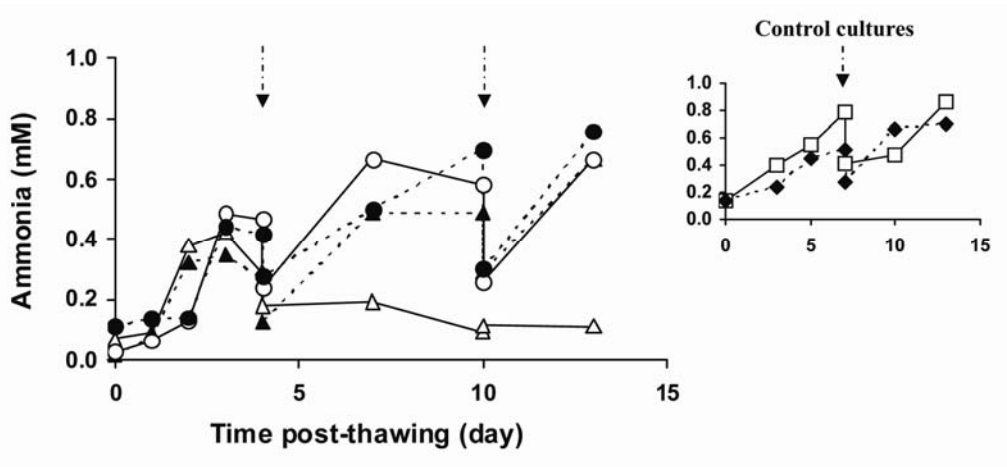
(ii)



(iii)



(iv)



(B)

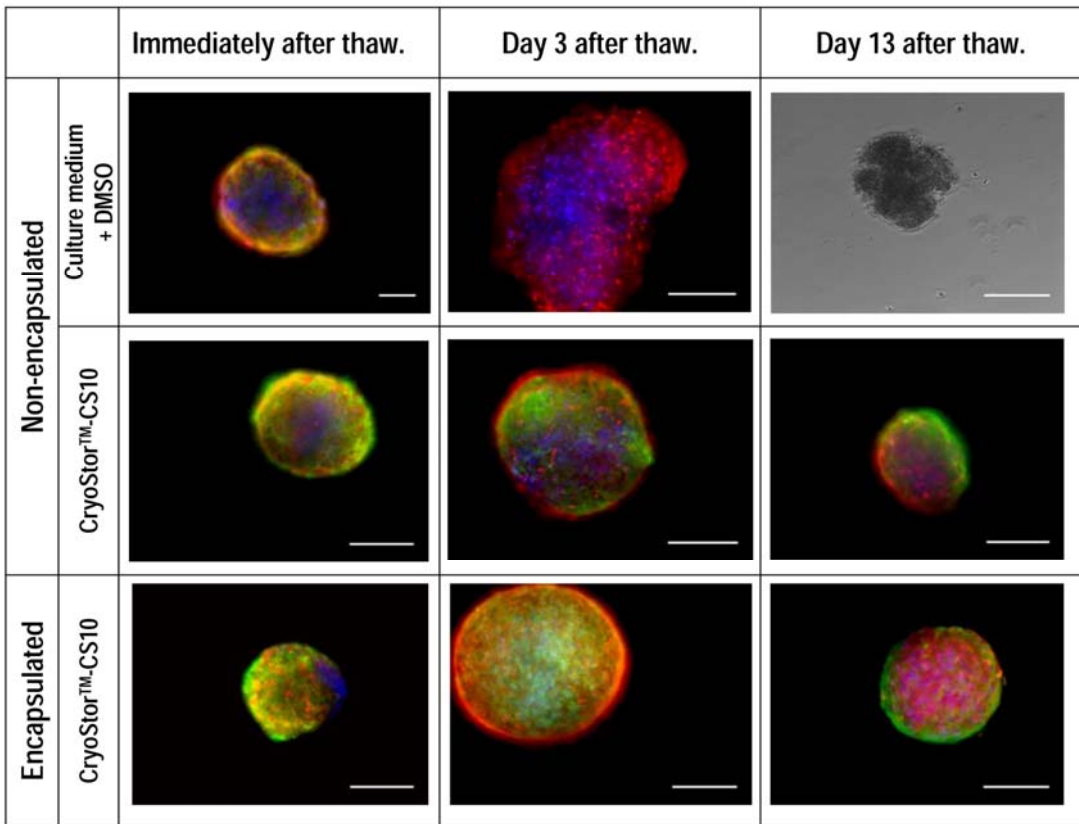


Figure 7. Post-thawing characterization of non-encapsulated and encapsulated neurospheres cultures. **A:** Profiles of glucose (i), lactate (ii), glutamine (iii) and ammonia (iv) concentrations in the supernatant of non-encapsulated neurospheres cryopreserved in culture medium (white triangles) or CryoStor™-CS10 solution (white circles) and encapsulated neurospheres cryopreserved in culture medium (black triangles) or CryoStor™-CS10 solution (black circles). Smaller charts show the profiles for non-cryopreserved control cultures of non-encapsulated (white squares) and encapsulated (black diamonds) neurospheres. Arrows represent 50% culture medium replacements; **B:** Immunofluorescence labelling of glial fibrillary acidic protein (green staining) and β -III Tubulin (red staining) immediately, 3 and 13 days after thawing. Blue staining - 4'-6-Diamidino-2-phenylindole (DAPI) binding to DNA. Scale bars = 200 μ m.

Table II. Effect of alginate encapsulation and cryopreservation medium on the ratios of produced lactate per consumed glucose in neurospheres cultures. SP / SP-ENC - non-encapsulated / encapsulated neurospheres cultures; CM - culture medium; CS10 - CryoStorTM-CS10 solution.

Time period (days)	Ratio Lac/Glc			
	SP-CM	SP-CS10	SP-ENC-CM	SP-ENC-CS10
19 - 23	0.0	1.0 ± 0.6	0.6 ± 0.3	0.7 ± 0.3
23 - 29	0.2 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	0.6 ± 0.2
29 - 32	0.0	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.2

A similar trend was obtained when neurospheres cell composition and cell specific interactions were evaluated (assessed by metabolic parameters and immunohistochemistry) (Table 2 and Figure 7); i.e for encapsulated neurospheres, both cryopreservation solutions allowed for the recovery of cell metabolism and cell-cell interactions. The same was observed for non encapsulated neurospheres cryopreserved in CryoStorTM-CS10. On the contrary, non-encapsulated neurospheres cryopreserved in cell culture medium resulted in neuronal cells death within 3 days post-thawing (Figure 7) and consequent inhibition of neurospheres metabolism.

For control cultures, from days 19 to 32, the lactate and glucose profiles as well as the calculated ratios Lac/Glc (average of 1.0), resemble neurons and astrocytes cocultures: consumption rates of glucose are high as well as lactate release as astrocytic population starts to proliferate and mature. Glutamine uptake is reduced due to the increase in astrocytic population. As seen in Figure 6, except for non-encapsulated neurospheres in cell culture medium, metabolism of cryopreserved samples followed the profiles of glucose and glutamine consumption and lactate and ammonia production found for control cultures.

Overall, post-thawing results show that the use of serum-free CryoStorTM-CS10 solution combined with UHV alginate encapsulation is the best option for the cryopreservation of brain cell neurospheres, allowing for a full recovery of

metabolic activity, functionality and maintenance of neurons-astrocytes interactions.

4. DISCUSSION

The aim of this study was to establish an improved cryopreservation protocol for primary brain neurospheres. Our strategy consisted in encapsulating the neurospheres within UHV alginate hydrogel to avoid post-thaw cell death and disaggregation. Our results show that the combination of cell entrapment in UHV alginate micro-capsules and serum-free CryoStorTM cryopreservation solution results in an optimum protocol for the cryopreservation of primary brain cell neurospheres. Such protocol allows for the maintenance of neurospheres morphology and single-cell membrane integrity, metabolic activity, cell composition and cell-specific interactions, which are major requirements for their post-thaw applications.

Besides the numerous factors known to influence the cryopreservation of single-cells, including cell size, shape and permeability to water and CPA, cryopreservation of cell spheroids deals with additional concerns related with heat and mass diffusion restrictions within the spheroid (Karlsson et al. 1996). Ice crystals can intercalate the tissue and mechanically deform cells, and ice may form inside cells, thereby disrupting the intercellular interactions that are needed to maintain the functional 3-D tissue architecture of spheroids (Karlsson et al. 1996). Studies on the cryopreservation of neurospheres are scarce and cryoprotocols applied to date are mostly crude and often adopted from those used for unrelated cell types (Paynter 2008). Most studies report the presence of partly broken spheroids post-cryopreservation and a decrease in cell yield although the extent of this loss varies (Purcell et al. 2003; Paynter 2008; Miranda et al. 2009). To overcome such problems, our strategy consisted in entrapping neurospheres within UHV alginate micro-capsules

Alginate encapsulation is a well established method for immobilization of cells in medical biotechnology (Clayton et al. 1993; Agren 1996; Hasse et al. 1997; Zimmermann et al. 2000) and has previously been reported as a strategy for improving the post-thaw cell viability and function of cell types which were shown to poorly survive such process, such as hepatocytes (Koebe et al. 1990; Hubel et al. 1991; Borel Rinkes et al. 1992; Dixit et al. 1993; Guyomard et al. 1996; Rialland et al. 2000; Birraux et al. 2002; Mahler et al. 2003) and pancreatic islets (Inaba et al. 1996; Zhou et al. 1997; Yoshifumi et al. 2001; Zimmermann et al. 2007). These aggregates may be further cultured inside alginate or used for its transplantation into animal models (Zimmermann et al. 2005). If necessary, alginate may also be dissolved for further applications post-cryopreservation by using Na_2SO_4 without compromising cell morphology or function (Malpique et al. 2009).

Some of the reported studies on the cryopreservation of encapsulated cells refer to an effect of the cryopreservation process on the capsules integrity and report a percentage of deformed or broken capsules following cryopreservation (Guyomard et al. 1996; Canaple et al. 2001; Song et al. 2005). In the present study, alginate concentration, polymerization time with cross-linking ion and encapsulation process were tested for their effect on the cryopreservation outcome of both the capsules and the cells inside them as these parameters are known to influence micro-capsules rigidity and mechanical stability (Zimmermann et al. 2005). With the optimized conditions, no capsule breakage was observed after cryopreservation, i.e., capsules shape and average diameter were maintained immediately after thawing and during the following 13 days in culture (data not shown). Furthermore, our results show that the encapsulation parameters tested were shown to greatly influence the post-thaw viability of the cell neurospheres and must be carefully tuned in order to achieve the best results. Capsules formed with an alginate concentration of 0.4% in NaCl polymerized by 20 min incubation were shown to yield the highest post-thaw recovery for either single cells or cell neurospheres formed after 7 days of culture

inside the micro-capsules. Thus, these conditions were selected for the encapsulation of primary brain cell neurospheres.

Initial culture conditions to establish viable cultures of primary rat neurospheres were based on previously reported optimized parameters (Moreira et al. 1995; Santos et al. 2007). Encapsulation of neurospheres was performed at the 5th day of culture to allow cell recovery from the shear force effects involved in tissue dissociation and adaptation to the culture system, which take place during the first days of culture (Santos et al. 2007). Overall, results presented herein show that the encapsulation process does not affect the culture viability neither neurospheres development.

Cryopreservation was performed at the 19th day of culture as our preliminary data have shown that more mature, differentiated spheroids (15-20 days culture), which form stronger cell-cell contacts, are able to survive the cryopreservation process better than those at 5 – 7 days *in vitro* (data not shown).

Evaluation of the post-thaw viability of cryopreserved spheroids is difficult due to the lack of common standardized methods and the broad variety of aggregates systems with different functional properties that must be unaffected after cryopreservation (e.g. albumin secretion of hepatocytes and glucose levels for Langerhans' islets). Furthermore, assessment of single-cells viability within the whole aggregates is intricate and its post-thaw dissociation is not a solution as cells may be damaged during enzymatic or mechanical destruction of the extracellular matrix and/or cell-cell contacts. Dead cells can be completely destroyed and thus will overestimate viability. Finally, such assays give no indication of survival of specific cell types and hence give no indication of the functionality of the tissue. Thus, in the present study, measurement of cell metabolic activity, concerning glucose and glutamine consumption and lactate and ammonia production, and metabolization of the alamarBlue assay substrate, were chosen for evaluation of cell recovery during 13 days of culture post-thawing. Moreover, cell membrane integrity within the aggregates and

immunocytochemical characterization of two major cell types, neurons and astrocytes, were performed without disruption of the aggregate through fluorescence microscopy.

This study demonstrates that micro-encapsulation within UHV alginate avoids the cryoinduced physical rupture of primary rat neurospheres as reduced fragmentation and improved maintenance of aggregates spherical shape were observed following cryopreservation. Furthermore, cell viability immediately post-thawing and during the following 13 days in culture is significantly increased when compared with non-encapsulated neurospheres. The metabolic activity of such cultures evidences specific neuronal-astrocytic enriched culture behaviour, comparable to unfrozen control cultures, which is a major requirement for post-cryopreservation use of such cell-systems. Thus, the presence of the gel not only avoids aggregates fragmentation but also mitigates cryopreservation-induced cell death, which has already been described for other cell types (Koebe et al. 1990; Inaba et al. 1996; Zhou et al. 1997; Rialland et al. 2000; Yoshifumi et al. 2001; Mahler et al. 2003; Malpique et al. 2009). Although the mechanisms underlying the hydrogel's cryoprotective action have not it been clearly elucidated, associations with several factors, such as the influence of the polymeric matrix on extracellular ice formation (Murase et al. 1997; Murase et al. 1999) or initiation of intracellular ice formation (Mazur 1965; Mazur et al. 1972; Toner et al. 1993), protection against apoptosis (Koebe et al. 1990; Hubel et al. 1991; Ji et al. 2004), or buffering for the CPA's diffusion (Tan et al. 1998) have been reported. Furthermore, the observed cryoprotective effect conferred through encapsulation may be related to the reduction in aggregates average diameter (approximately 200 μm) when compared to non-encapsulated cultures as it is known that heat and mass (water and CPA) diffusion restrictions in large spheroids (>350 μm) result in varying cryoprotection achieved from the surface to the centre of the spheroid, thus leading to cryodamage (Karlsson et al. 1996; Elmoazzen et al. 2005). Heat and mass diffusion restrictions and ice front migration within the

alginate matrix are also known to impact the cryopreservation outcome of encapsulated cells (Karlsson et al. 1996; Cui et al. 2002). In the future, more fundamental studies on the physico-chemical and bio-physical phenomena occurring during freezing/thawing of encapsulated neurospheres will allow for a further improvement of this process.

In what concerns the cryopreservation solution, the use of CryoStorTM improves cryopreservation and post-thawing recovery for both non-encapsulated and encapsulated neurospheres compared to standard culture medium as high post-thaw membrane integrity and metabolic were maintained immediately after thawing and during the following 13 days in culture, either with or without alginate gel entrapment. It was shown that the combination of UHV alginate entrapment and CryoStorTM solution significantly contributed to improved cryopreservation outcome as has already been described for cell monolayers (Malpique et al. 2009). CryoStorTM has been developed from the platform hypothermic storage solution HypothermosolTM, which is part of a number of preservation solutions currently available in the field of regenerative medicine (Taylor et al. 1995; Baust et al. 1998). These solutions have been carefully formulated to maintain the ionic and hydraulic balances of cells at low temperatures thus facilitating the preservation of cell homeostasis and control of the ionic environment that cannot be achieved using traditional preservation media formulations consisting of a basal culture media with serum protein and DMSO supplementation (Taylor et al. 1985; Southard et al. 1990; Basora et al. 1999; Taylor et al. 2001).

To our knowledge this is the first report on the successful culture of primary brain neurospheres inside UHV alginate micro-capsules without affecting neurospheres metabolism and differentiation patterns in culture. Furthermore, it is the first report on the successful cryopreservation of primary brain neurospheres with 24 hours post-thaw viabilities above 60% and full recovery of cell viability and metabolic activity after 3 days of culture post-thawing.

5. CONCLUSION

We show that micro-encapsulation within UHV alginate does not affect the culture of brain cell aggregates and is in fact an effective approach for their cryopreservation. Furthermore, the use of CryoStor™ solution provides further protection for non-encapsulated aggregates.

The ability to cryopreserve primary brain neurospheres for long periods will greatly alleviate the problems related with the time consuming and labour-intensive of neurospheres preparation and facilitate their widespread application as *in vitro* model systems for pharmacotoxicological applications, brain research and tissue engineering. Additionally, successful cryopreservation of primary brain aggregates would aid the implementation of tissue banks of neuronal stem cell spheroids for tissue engineering and clinical applications of spheroid-based support systems.

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CRYOPRESERVATION OF hESC

Novel Strategies towards High-Throughput Cryopreservation of hESC

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ABSTRACT

Human embryonic stem cells (hESC) hold tremendous potential in the emerging fields of gene and cell therapy and regenerative medicine. In addition they are also a useful tool in basic scientific research and cytotoxicity screening. One of the major challenges regarding the application of these cells is the development of an efficient cryopreservation protocol for hESC since current methods present poor recovery rates and/or technical difficulties which impair the development of a scalable, automated process that can handle bulk quantities of pluripotent cells for clinical or pharmacological applications (Reubinoff et al. 2001; Ji et al. 2004; Kim et al. 2004; Richards et al. 2004; Hunt et al. 2007).

The main focus of this work was the development of an effective, scalable, xeno-free cryopreservation protocol for hESC towards a high-throughput, GMP-compliant process. Within this goal, we have investigated combined strategies for the cryopreservation of hESC colonies. Slow-rate freezing of intact hESC colonies or colony fragments (clumps) was evaluated and compared with a novel surface-based vitrification of adherent colonies. Since the maintenance of colonies/clumps integrity is a critical requirement for their successful cryopreservation, entrapment within ultra-high viscous (UHV) alginate was evaluated as the main strategy to avoid the commonly observed loss of viability and colony fragmentation during slow-rate freezing. As the cryomedium, serum-free CryoStorTM solution was compared with serum-free and serum-supplemented culture medium (CM), all containing 10% dimethyl sulfoxide (DMSO).

The results obtained indicate that entrapment beneath a layer of UHV alginate does not provide further protection to hESC cryopreserved through slow-rate freezing, whether CM or CryoStorTM solution are used as the cryomedium.

On the contrary, vitrification of adherent hESC colonies on the top of hFF feeders in gelatine-coated culture dishes yielded significantly higher recovery rates when compared with the slow-rate freezing approach. We show that the

pluripotency of hESC was not changed after a vitrification/thawing cycle and during further propagation in culture. This novel surface-based vitrification method may facilitate the development of a high-throughput cryopreservation process for hESC and reduce the time required to amplify frozen stocks thus supporting the widespread use of these cells in research, clinical or pharmacological applications.

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1. INTRODUCTION

hESC are pluripotent cells derived from the inner mass of the blastocyst (Thomson et al. 1998). Due to their ability for continuous self-renewal during extended periods and their capacity to give rise to multiple types of differentiated cells from all three embryonic germ layers, hESC hold tremendous potential in fundamental scientific research (Eisenberg et al. 2004; Chen et al. 2006), drug discovery and toxicology (Rohwedel et al. 2001; Gribaldo 2002) as well as in the newly emerging fields of gene therapy (Strulovici et al. 2007), tissue engineering and cellular therapy (Gerecht-Nir et al. 2004; Lerou et al. 2005).

One of the major challenges regarding the application of these cells is the production of cell banks of well-characterized and safety-tested stocks of hESC. This requires the implementation of efficient, reproducible, scalable and automatable methodologies that facilitate the cryopreservation of sufficient cell numbers. Such protocols must assure high survival, low colony fragmentation, low differentiation rates and maintenance of pluripotency post-thawing (Hunt et al. 2007). Furthermore, for standardisation and quality control of cell banking, such protocols should be compatible with current good manufacturing practice (GMP) (Stacey et al. 2007), which implies avoiding animal-component products as well as freezing methods involving direct contact with liquid N₂ (LN₂) in order to reduce the potential transmission of infectious agents into the cells (Hunt et al. 2007).

In general, two techniques have been applied for hESC cryopreservation, slow-freezing-rapid thawing (Ji et al. 2004; Ha et al. 2005; Wu et al. 2005; Ware et al. 2007; Lee et al. 2008; Martin-Ibanez et al. 2008) and vitrification of colonies in suspension (Reubinoff et al. 2001; Kim et al. 2004; Richards et al. 2004; Hunt et al. 2007; Li et al. 2008).

The conventional slow-freezing and rapid thawing in cryovials using DMSO as the cryoprotectant (CPA) (Grout et al. 1990) is efficient for the cryopreservation of murine and porcine embryonic stem cells (mESC) (Robertson 1987; Shim et al.

1997) but when applied to hESC results in low post-thaw survival, low plating efficiencies, high differentiation rates and loss of pluripotential capacity, presumably due to ice crystal formation that disrupts cell-cell adhesion (Reubinoff et al. 2001; Ji et al. 2004; Richards et al. 2004).

Vitrification, which works on the principle of glass induction instead of ice crystal formation, uses high CPA concentrations together with flash-freezing in LN₂ to minimize cell exposure to non-physiological temperatures and circumvents problems associated with ice formation and cell dehydration. Vitrification has come to be considered the preferred cryopreservation technique for hESC since it was first reported by Reubinoff *et al.* (Reubinoff et al. 2001; Hunt et al. 2007). Despite yielding higher plating efficiencies and lower differentiation rates when compared to slow-rate freezing (Reubinoff et al. 2001; Kim et al. 2004; Richards et al. 2004; Zhou et al. 2004), vitrification protocols for hESC clumps are labour-intensive and extremely tedious to perform manually. Moreover, recovery rates are highly dependent on the operator's technical skills and on the cellular stresses that occur prior to freezing, including colonies processing into clumps. All this makes current vitrification protocols unsuitable for development of an automated, high-throughput process that can handle bulk quantities of hESC (Hunt et al. 2007).

Attempts at improving hESC cryopreservation conditions have focus on different strategies including slow-rate freezing of colonies adherent to culture dishes and entrapped within matrigel (Ji et al. 2004), trehalose loading (Ji et al. 2004; Wu et al. 2005), the addition of collagen and laminin to the cryopreservation medium (Kim et al. 2004), and more recently cryopreservation in the presence of caspase inhibitors (Heng et al. 2007) or the Rho-associated kinase (ROCK) inhibitor (Martin-Ibanez et al. 2008).

In the present study, we aimed to develop an efficient, reproducible, GMP-compliant cryopreservation method for hESC, which allowed for process scalability and automation. Within this goal, combined strategies for the

cryopreservation of hESC through the standard slow-rate freezing/rapid thawing protocol were investigated based on our previously studies with adherent monolayers and 3-D aggregates (Malpique et al. 2009) and reports in literature on different strategies to improve the cryopreservation of hESC (Ji et al. 2004; Richards et al. 2004). Slow-rate freezing of intact hESC colonies or colony fragments (clumps) was evaluated and compared with a novel surface-based vitrification of adherent colonies, which eliminates the cell-clump selection and overcoming the technical problems associated with the vitrification of hESC clumps (Ji et al. 2004; Heng et al. 2005).

As the main strategy for avoiding the commonly observed cell death and colony fragmentation that results from slow-rate freezing hESC, we have evaluated cell entrapment within clinical-grade, xeno-free, UHV alginate since its ability to improve post-thaw cell viability of differentiated cells monolayers and functional brain neurospheres has been recently described by our group (Malpique et al. 2009; Malpique et al. 2010). Additionally, to further potentiate the advantages of alginate encapsulation, standard cell culture media was compared with protein- and serum-free CryoStorTM solution as the vehicle solution for the CPA (DMSO), which has been reported to significantly enhance post-thaw cell survival and recovery in a series of cellular systems (Elford et al. 1972; Baust et al. 2001; Sosef et al. 2005; Stylianou et al. 2006; Malpique et al. 2009). Finally, the effect of the addition of the Rho-kinase inhibitor ROCK and apoptosis inhibitor Z-VAD-FMK to the cryo- and post-thaw media was tested as these have already been shown to improve post-thaw recovery of hESC (Heng et al. 2007; Martin-Ibanez et al. 2008).

2. MATERIALS AND METHODS

2.1. MATERIALS

VitroHES was obtained from Vitrolife (Göteborg, Sweden), trypsin-EDTA (1x solution) (0.25%), TrypLE Select, phosphate buffer saline (PBS), non-essential amino-acids, fetal bovine serum (FBS) and alamarBlue were obtained from Invitrogen (Paisley, UK). Basic fibroblast growth factor (bFGF) was obtained from Peprotech (Neuilly-Sur-Seine, France). Mitomycin C, gelatin from porcine skin A, β -mercaptoethanol, DMSO, and the fluorescent dyes, fluoresceine diacetate and ethidium bromide, were obtained from Sigma-Aldrich (Steinheim, Germany). CryoStorTM-CS10 was obtained from BioLife Solutions (Bothell, WA, USA). Culture plates were obtained from Sarstedt (Nümbrecht-Rommelsdorf, Germany). Cell strainers and the caspase inhibitor Z-VAD-FMK were obtained from BD Biosciences (San Jose, CA, California). ROCK inhibitor Y-27632 was obtained from Calbiochem, Merck (Nottingham, UK). The alkaline phosphatase detection kit was obtained from MilliporeTM (Billerica, MA, USA). Other chemicals were of the purest grade available from regular commercial sources.

Alginates: Clinical grade, UHV alginates were produced and purified from stipes of *L. nigrescens* and *L. trabeculata* as described elsewhere (Zimmermann et al. 2001). A 1:1 mixture of purified alginates was used at 0.4% or 0.7% (w/v) in 0.9% NaCl solution.

2.2. hESC CULTURE

SA461hESC line (Cellartis AB, Göteborg, Sweden) was maintained on (mitotically) mitomycin-C-inactivated human foreskin fibroblasts (ihFF) (ATCC collection, Cat. No. CRL-2429) in hESC culture media, VitroHES supplemented with 0.01 μ g/ml bFGF. Depending on the desired application, cells were passaged by digestion with TrypLE Select for 6-8 minutes of colonies of undifferentiated hESC and transference of single cells to fresh feeders at approximately 10- to 12-day

intervals, i.e., when the hESC colonies covered about 75-85% of the surface area of the culture well) (Ellerström et al. 2007).

2.3. ENTRAPMENT OF HESC COLONIES BENEATH AN UHV ALGINATE LAYER

After 7 days of culture following serial passage (hESC covering 60-70% of the surface area of the culture plate), an UHV alginate gel layer (0.7% (w/v) in NaCl) was added on top of the cells. The culture medium was removed from the wells and cells were rinsed in phosphate-buffered saline (PBS). An incubation step with poly-L-lysine for 25 minutes was then performed to allow the adhesion of alginate to the well's surface. After washing again with PBS, an UHV alginate gel layer (approximately 500 µl/well) was added over the cells. Alginate gelling was achieved through 25 minutes incubation with 20 mM BaCl₂ solution adjusted to 290 mOsm using NaCl buffered at pH 7.2 by 5 mM histidine followed by three times washing with PBS with 0.5 mM MgCl₂. Whenever required, alginate was dissolved by incubating with a 20 mM Na₂SO₄ solution for 20 minutes at 37°C in a humidified atmosphere of 5% CO₂ in air.

Adherent hESC in culture without an alginate cover were used as control.

2.4. ENCAPSULATION OF HESC CLUMPS WITHIN UHV ALGINATE

After 12 days of culture following serial passage, hESC were mechanically cut into small clumps and encapsulated by resuspending the pellet in 3 ml of UHV alginate (0.4% (w/v) in NaCl).

Micro-capsules formation was achieved by using the conventional air-jet two-channel droplet generator as described in detail elsewhere (Zimmermann et al. 2001), yielding capsules with a diameter of approximately 200-400 µm. For cross-linkage of the alginate, capsules were dropped directly from the droplet generator's nozzle into a 20mM BaCl₂ solution, adjusted to 290mOsm using NaCl buffered at pH 7 with 5mM histidine.

hECS-containing alginate micro-capsules were polymerized for 20 minutes, washed three times with 0.9% NaCl solution and cultured on inactivated hFF in culture plates. Non-encapsulated hESC clumps were cultured under the same conditions as a control.

2.5. CELL CRYOPRESERVATION

After 8 days of culture, hESC were cryopreserved as intact, confluent, adherent colonies on hFF feeders, by either slow-rate freezing or vitrification. hESC clumps were also cryopreserved, by slow-rate freezing, after 1 or 6 days of culture inside the micro-capsules, in suspension inside cryovials.

2.5.1. Slow-Rate Freezing

Adherent hESC Colonies: At the moment of freezing, culture medium was removed and 750 μ l cryomedium was added to each plate as this was determined to be the minimal amount of liquid which allowed faster thawing while covering completely the area of the culture dish.

hESC Clumps in Suspension: Encapsulated and non-encapsulated hESC clumps were harvested from the culture plates after 1 or 6 days of culture post-encapsulation and centrifuged (400 *g*, 5 minutes). The pellet from each culture was resuspended in each of the cryopreservation media tested and cell suspension was transferred to 1 ml cryovials.

For the cryopreservation of both adherent and encapsulated hESC, three cryomedia were tested: serum-free culture medium VitroHES (CM); CM containing 50% FBS (CM-FBS) and CryoStorTM-CS10, all containing 10% (v/v) DMSO. For adherent hESC, the cryomedium was further supplemented with 100 μ M of Z-VAD-FMK or ROCK inhibitor Y-27632 or both.

The cells were allowed to equilibrate in the cryopreservation medium for 20 minutes at 4°C. Cooling was achieved with a rate-controlled freezing system

(Planer Kryo 560-16, Planer, Middlesex, UK) using a cooling rate of 1°C per minute. Samples were stored in the vapour-phase of LN₂ for 1 week.

2.5.2. Vitrification

Two serum-free vitrification solutions (VS) were used, both based on CM: VS1 included 10% DMSO and 10% ethyleneglycol (EG); VS2 contained 0.5 M sucrose, 20% DMSO and 20% EG. Adherent colonies were incubated in VS1 for 1 minute followed by VS2 for 5 sec. As hESC colonies possess a relatively low surface area to volume, the rapid cooling and warming rates required for vitrification are difficult to achieve (Heng et al. 2005). Thus, in order to facilitate rapid cooling (>1000°C/second), VS2 was completely aspirated from the wells immediately before plunging into LN₂. After 10 sec in LN₂, culture plates were transferred to the vapour-phase of LN₂ and stored for 1 week before thawing. During transfer from LN₂ recipient to the vapour-phase of LN₂, adherent colonies were covered by LN₂ to avoid devitrification.

2.5.3. Thawing

CPA dilution was performed for slow-rate frozen and vitrified samples by incubation in two warming solutions (WS): WS1, consisting of 0.4 M sucrose in CM, and WS2, consisting of 0.1 M sucrose in CM.

Slow-rate frozen samples were quickly thawed in a 37°C water bath. Stepwise dilution of CPA was then performed using the two. To minimize osmotic shock to the cells, the cryopreservation solution within each culture plate was gradually diluted by dropwise addition of 750 µl WS1 and incubation for 1 minute, followed by 5 minutes incubation in 1.5 ml WS2. For hESC clumps, CPA dilution was performed by transferring the clumps from the cryovials to 40 µm cell strainers, which were placed inside 9.6 cm² culture plates, and then adding the WS. Cells were then returned to culture conditions in hESC culture media. Adherent hESC cultures were supplemented 100 µM of Z-VAD-FMK or 100 µM of ROCK inhibitor Y-27632 or both.

Vitrified samples were quickly thawed by quick transfer from the vapour-phase of LN₂ to the laminar flow and immediate incubation for 1 minute in 2x diluted WS1, followed by 5 minutes incubation in WS2. During transfer from the vapour-phase of LN₂ in storage tank to the laminar flow, adherent colonies were covered by LN₂ to avoid devitrification.

2.6. POST-THAW SURVIVAL AND CHARACTERIZATION OF hESC COLONIES

After thawing, cryopreserved hESC were maintained in culture for at least 4 days for assessment of cell survival. Whenever possible, cells were further maintained in culture for several passages for post-thaw studies of growth and pluripotency. Adherent hESC were maintained on the ihFF feeder layer on which they were frozen while hESC clumps were transferred to fresh feeders on gelatine-coated 9.6 cm² culture plates.

2.6.1. Assessment of hESC Survival

Viability of control (non-frozen) and frozen/thawed hESC colonies was assessed at day 0, day 1 and day 4 post-thawing (corresponding to 7, 8 and 12 days of culture for control samples, respectively), both through qualitative assessment of the plasma membrane integrity and quantitative measurement of cell metabolic activity. Adherent colonies and clumps were furthered monitor for morphological assessment of colony/clump integrity and attachment to the culture surface using phase-contrast microscopy (Leica Microsystems GmbH, Wetzlar, Germany).

2.6.1.1. Membrane Integrity Assay

hESC colonies on culture plates were stained with a dual fluorescence test using the enzyme substrate fluorescein diacetate and the DNA-dye ethidium bromide as described in the literature (Dankberg et al. 1976) for assessment of

membrane integrity. Samples were imaged using fluorescence microscopy (Leica Microsystems GmbH, Wetzlar, Germany).

2.6.1.2. Metabolic Activity Assay

hESC metabolic activity was assessed using the non-toxic metabolic indicator alamarBlue following the manufacture's recommendation. Briefly, hESC colonies on culture plates were incubated with fresh medium containing 10% alamarBlue and 6 hours later fluorescence was measured in 96-well plates using a microplate fluorescence reader (Biotek Instruments, Winooski, VT, USA).

2.6.2. hESC Pluripotency

2.6.2.1. Alkaline Phosphatase (AP) Staining

Vitrified/thawed hESC were analysed 4 days post-thawing (P0) and after 1 (P1) and 2 (P2) cell passages post-thawing. Cultures were stained using an AP activity detection kit according to the manufacturer's instructions and observed under phase-contrast microscopy (Leica Microsystems GmbH, Wetzlar, Germany). Positively stained hESC colonies were considered as survived, undifferentiated colonies.

2.6.2.2. Immunodetection of Pluripotency Markers

The presence of hESC cell surface markers SSEA-4 and TRA-1-60 and the transcription factor OCT-4 was determined by immunohistochemistry and flow cytometry after 4 days post-thawing and after 1 and 2 cells passages post-thawing. The primary antibodies used were specific for SSEA-4, OCT-4 and TRA-1-60 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). As secondary antibodies, Alexa-conjugated goat anti-mouse immunoglobulin M (IgM) and Alexa-conjugated goat anti-mouse IgG (all from Invitrogen, Paisley, UK) were used. Human foreskin fibroblasts and hESC without primary antibodies were used as negative controls.

Immunohistochemistry: hESC cultures were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 20 minutes, permeabilized (only for detection of intracellular marker OCT-4) for 5 minutes in 0.5% Triton X-100 solution (Sigma-Aldrich) and subsequently incubated with primary antibody diluted (1:20) in 0.125% fish skin gelatine in PBS overnight at 4°C. Cells were washed three times in washing buffer (5% FBS in PBS) and the secondary antibodies (diluted 1:200 in 0.125% fish skin gelatine in PBS) were applied to the cells for 60 minutes at room temperature in the dark. After three washes in washing buffer, cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Cells were visualized through fluorescence microscopy (Leica Microsystems GmbH, Wetzlar, Germany).

Flow Cytometry: hESC colonies were then harvested by enzymatic dissociation, resuspended in washing buffer and filtered through a 40 µm strainer. Cells were fixed and permeabilized with BD Cytofix/Cytoperm (only for detection of intracellular marker OCT-4) solution during 15 minutes at room temperature and incubated in primary antibodies solution (dilution 1:50) for 1 hour at 4°C, washed three times in washing buffer and then incubated in secondary antibodies solution (dilution 1:200) for 30 minutes at 4°C. After washing twice, cells were suspended in washing buffer and the percentage of undifferentiated, pluripotent hESC was determined by flow cytometry (CyFlow-space, Partec GmbH, Münster, Germany).

2.6.2.3. Embryoid Bodies (EB) Formation

EB were formed by culturing aggregates of hESC in suspension for 1 week in CM without bFGF before harvesting and culture in gelatin-coated plates for further 2 weeks in CM without bFGF (media change every second day). Cells were then assessed for spontaneous differentiation into derivatives of all three embryonic germ layer by immunostaining with markers for mesoderm (α -smooth muscle actin – ASMA), endoderm (forkheadbox A2 - FOXA2) and ectoderm (Nestin and β_{III} -tubulin) as described in section 2.6.2.2. Primary antibodies and

dilutions used were: anti-ASMA (1:200, from Dako, Glostrup, Denmark), anti-FOXA2 (1:500, from Santa Cruz Biotechnology, Santa Cruz, CA, USA), nestin (1:200, from Millipore, Billerica, MA) and anti- β_{III} -tubulin (1:200, from MilliporeTM, Billerica, MA, USA). OCT-4 immunostaining was used as a control for pluripotent cells. Samples were imaged using fluorescence microscopy (Leica Microsystems GmbH, Wetzlar, Germany).

2.7. DATA PRESENTATION/ANALYSIS

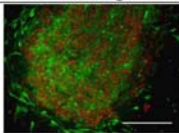
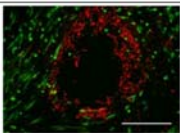
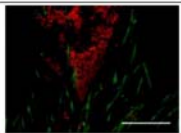
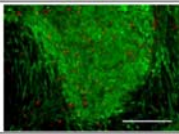
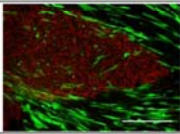
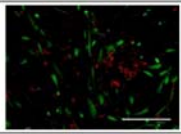
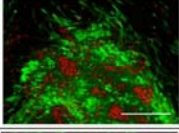
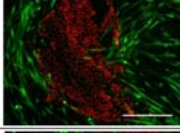
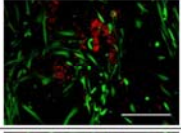
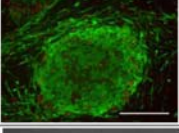
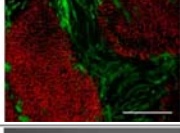
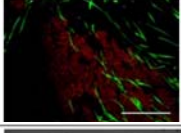
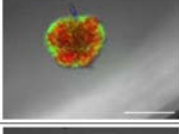




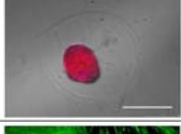
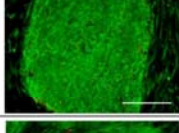
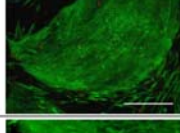
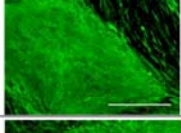
All data presented corresponds to $n \geq 6$ experimental replicates (minimum of 2 experimental repeats with 3 sample replicates per experiment). Error bars denote the standard deviation of the mean. Fluorescence units were converted to percent recovery based upon non-cryopreserved experimental controls (mean \pm SEM). For membrane integrity, immunocytochemistry and SEM assays representative photographs are depicted.

3. RESULTS

3.1. POST-THAW SURVIVAL OF hESC

To evaluate the viability of hESC, control (non-frozen) and thawed hESC colonies on culture plates were first assessed for membrane integrity and metabolic activity (Figure 1). Adherent colonies and clumps were furthered monitor for morphological assessment of colony/clump integrity and attachment to the culture surface.

(A)

Slow-Rate Freezing			Immediately post-thawing	1 day post-thawing	4 days post-thawing
Colony Fragments (Clumps)	Adherent Colonies				
	CM + 10%DMSO	CM + 10%DMSO + Alginate			
	CryoStor™-CS10	CryoStor™-CS10 + Alginate			
	CM + 10%DMSO	CM + 10%DMSO + Alginate			
Vitrification in Surfaces	CM + 10%DMSO	CM + 10%DMSO + Alginate			
	CM + 10%DMSO + Alginate				
Non-Frozen Controls					

(B)

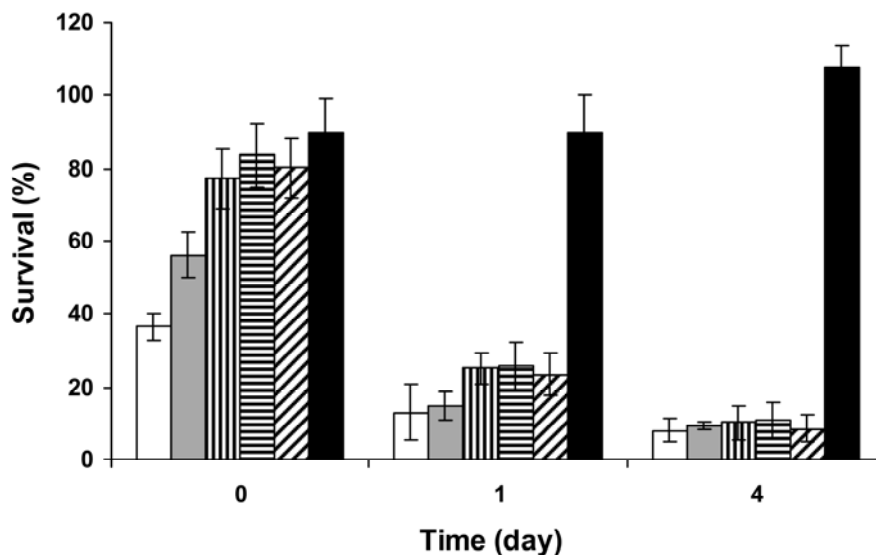


Figure 1. Post-thaw survival of adherent hESC colonies and hES clumps cryopreserved by slow-rate freezing and adherent hESC colonies cryopreserved by vitrification in serum-free culture medium. **A:** membrane integrity of cryopreserved cells immediately, 1 and 4 days after thawing. Green cells with undamaged membranes and metabolic activity; red cells with damaged membranes. Scale bars = 500 μ m. **B:** Post-thaw alamarBlue viability test. hESC were cryopreserved as adherent colonies by slow-rate freezing in CM (white bars), CM-FBS (grey bars) or CryoStor™-CS10 (vertical dashed bars). Cells were also frozen in CryoStor™-CS10 in the presence of ROCK and Z-VAD-FMK molecules (horizontal dashed bars) or with a layer of UHV alginate covering the cells (diagonal dashed bars). hESC colonies were also cryopreserved by vitrification (black bars). Survival is presented as a percentage of viability of control (non-cryopreserved) cultures.

3.1.1. Cryopreservation through Slow-Rate Freezing

3.1.1.1. Effect of the Cryomedium and Apoptosis Inhibitors

When hESC colonies on ihFF feeders were cryopreserved by slow-rate freezing, cell death and detachment from the surface started immediately after thawing and continued in the following hours. Within 24 hours post-thawing, the majority of the cells had detached and the ones that remained adherent had damaged membranes (Figure 1A) and/or were metabolically inactive as confirmed through alamarBlue assays (Figure 1B). For hESC clumps, cell death was observed

immediately after thawing and continued in the following days post-thawing. hESC clumps were not able to attach and proliferate in fresh ihFF feeders.

As seen in Figure 1, the addition of 50% FBS to the cryomedium or its replacement by the CryoStorTM solution had no effect on the cryopreservation outcome when compared with serum-free CM. Furthermore, no cryoprotective effect was observed when the apoptosis inhibitors Z-VAD-FMK and ROCK inhibitor Y-27632 were added to the cryo- or post-thaw medium for any of the concentrations tested (data not shown).

3.1.1.2. Effect of Entrapment within UHV Alginate

Before assessing the effect of UHV alginate entrapment on post-thaw cell recovery, its biocompatibility with the hESC was analysed and confirmed (data not shown). Alginate entrapment time was optimized for each system: alginate addition over adherent cells should be performed after achievement of confluent hESC colonies and micro-encapsulation of hESC clumps should be performed after cells recovery from the shear force effects involved in tissue dissociation so that cell proliferation is not affected. Thus, hESC colonies were entrapped beneath an alginate layer after 7 days of culture (corresponding to 24 hours prior to freezing) and hESC clumps were encapsulated after 24 hrs of culture in suspension in bFGF supplemented medium on ihFF feeders (corresponding to 1 or 6 days prior to freezing).

Our results show that UHV alginate entrapment does not provide protection to slow-rate frozen hESC colonies or clumps. Loss of membrane integrity in micro-encapsulated clumps occurred immediately after thawing, whether they were frozen 1 or 6 days after encapsulation. For hESC colonies, although reduced cell detachment from the culture surface was observed immediately after thawing, within 24 hours all cells presented damaged membranes (Figure 1).

3.1.2. Cryopreservation through Vitrification

Both membrane integrity assays and assessment of metabolic activity confirm that survival rates for vitrified samples were comparable to controls (Figure 1 and Figure 2A). Immediate post-thaw cell viability was found to be over 90% and no cell loss or decrease in viability was observed within the next 4 days.

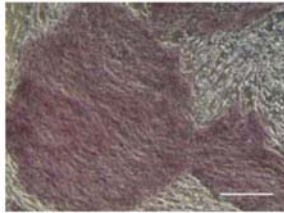
3.2. POST-THAW LONG-TERM CHARACTERIZATION OF hESC COLONIES

Vitrified/thawed hESC were characterized in detail after culturing for 3 additional passages. Cell survival and undifferentiated status of hESC colonies were confirmed through staining for AP 4 days post-thawing and after 1 and 2 cell passages (Figure 2).

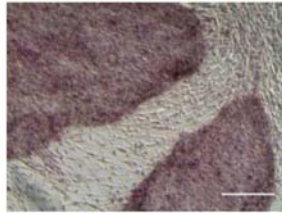
It was found, both through immunohistochemistry and flow cytometry analysis, that after vitrification, thawing, and culturing, hESC colonies maintained expression of undifferentiated hESC markers, such as SSEA-4, TRA-1-60 and Oct-4 comparable to control cultures (non-frozen) at the same passages (Figure 2B and 2C).

(A)

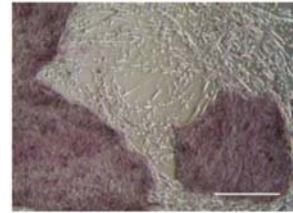
Control cultures (non-frozen) – day 12, P0



Vitrified cultures - 4 days post-thawing – day 12, P0

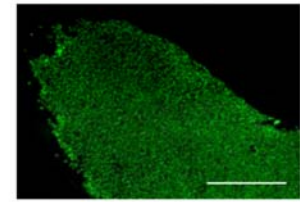
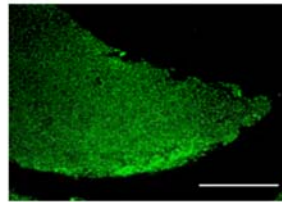
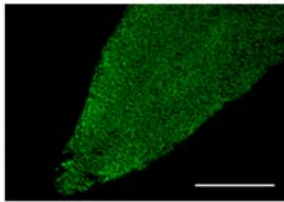


Vitrified cultures – day 12, P2

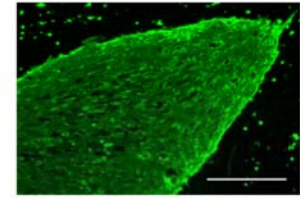
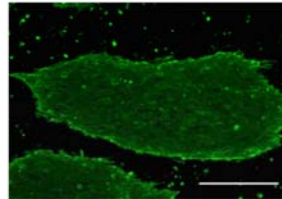
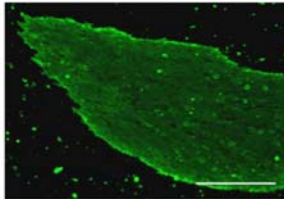


(B)

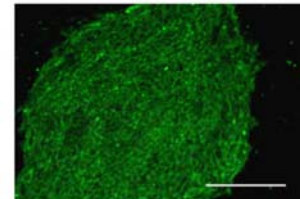
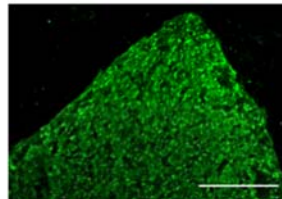
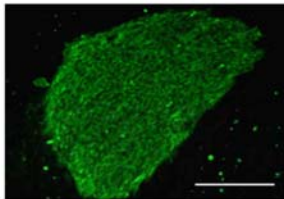
OCT-4



SSEA-4



TRA-1-60



(c)

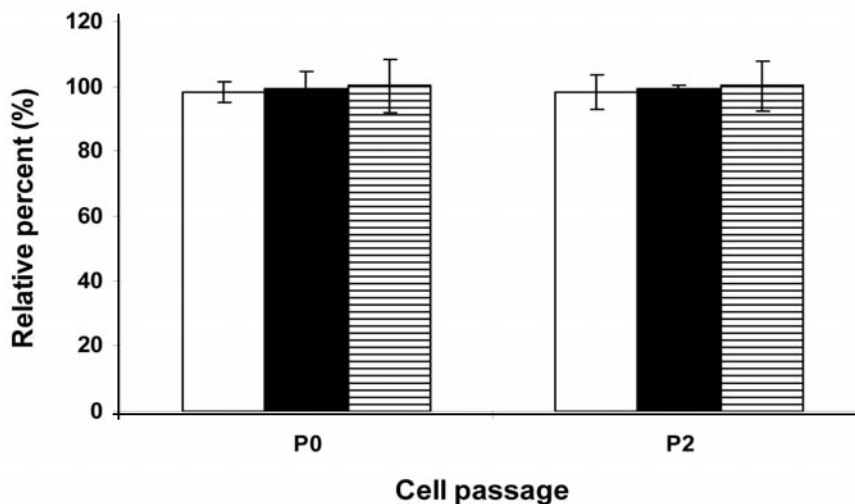


Figure 2. Characterization of vitrified/thawed cultures of hESC after 4 days of culture post-thawing (P0) and after two cell passages post-thawing (P2). **A:** Alkaline phosphatase staining. Scale bars = 500 μm ; **B:** Immunofluorescence labelling of whole hESC colonies with SSEA-4, TRA-1-60 and Oct-4 hESC markers. Scale bars = 500 μm ; **C:** Summary of the results of the flow cytometry analysis with the hESC markers SSEA-4 (white bars), TRA-1-60 (black bars) and Oct-4 (horizontal hatched bars). Results are presented as a percentage of each of the markers in non-cryopreserved control cultures.

Finally, it was shown by spontaneous differentiation into EB that vitrified-thawed hESC colonies expressed specific markers for all three germ layers – mesoderm (α -smooth muscle actin – ASMA), endoderm (forkheadbox A2 - FOXA2) and ectoderm (nestin and β_{III} -tubuline) - indicating that the ability of these cells to differentiate into progeny cells in each of the three embryonic germ layers was maintained (Figure 3). Oct-4 / SSEA-4 expression was not observed.

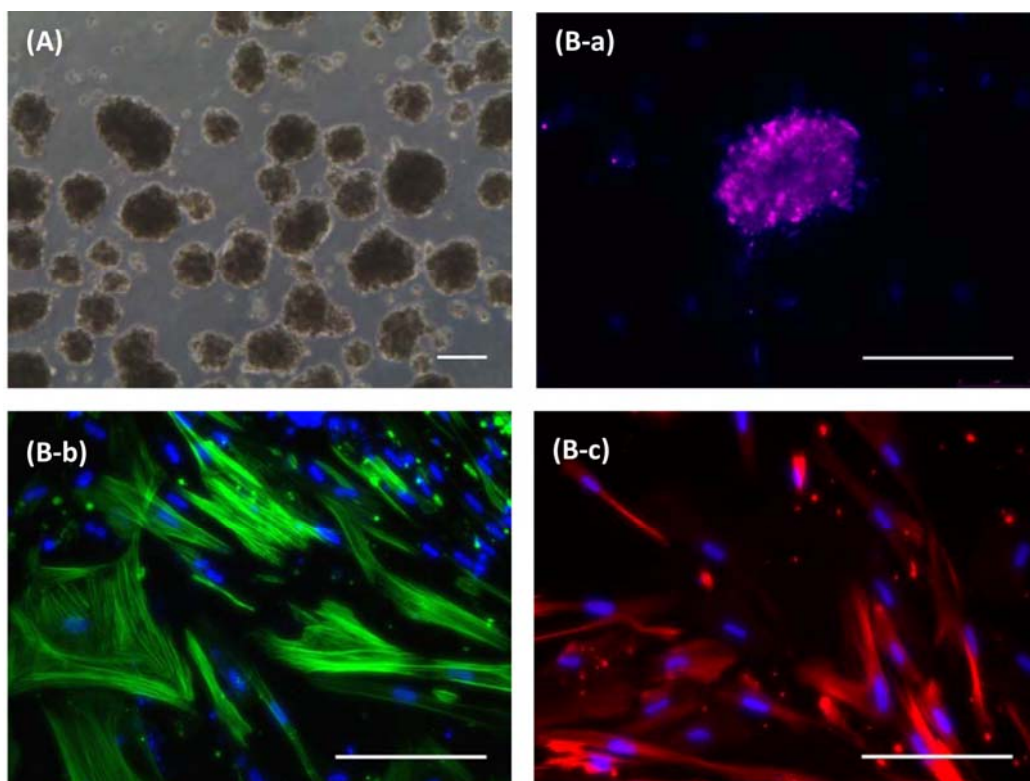


Figure 3. Pluripotency of vitrified/thawed hESC after two cell passages post-thawing. **A:** Phase contrast microscopy image of EB aggregates derived from vitrified/thawed hESC **B:** Immunofluorescence labelling of cells spontaneously differentiated from EB aggregates with markers of the three embryonic germ layers: (a) endoderm (forkheadbox A2 - FOXA2); (b) mesoderm (α -smooth muscle actin – ASMA) and (c) ectoderm (Nestin). Scale bars = 200 μm .

4. DISCUSSION

The aim of this study was to establish an improved cryopreservation protocol for hESC by investigating different strategies towards an efficient, scalable process. In this sense, slow-rate freezing and vitrification were tested as the freezing method for cryopreserving hESC colonies.

When non-entrapped hESC colonies on ihFF feeders were cryopreserved by slow-rate freezing, cell death and detachment from the surface started immediately after thawing and continued in the following hours: within 24 hours post-thawing, the majority of the cells had detached and the ones that remained adherent had damaged membranes and/or were metabolically inactive. This

results are in agreement with previous reports on the cryopreservation of adherent hESC by slow-rate freezing (Heng et al. 2006; Heng et al. 2007) as well as reports on loss of viability and excessive cell detachment for different cell lines cryopreserved as monolayers (Wusteman et al. 1997; Pasch et al. 1999; Ebertz et al. 2004; Malpique et al. 2009).

Although the mechanism is far from fully understood, several studies indicate that maintaining cell-cell contact improves hESC recovery following cryopreservation through slow-rate freezing (Ji et al. 2004; Hunt et al. 2007; Nie et al. 2009). However, cryopreservation of hESC as clusters or colonies brings additional concerns. Damage caused by intracellular ice propagation (IIF), either by random nucleation events within the cluster and propagation through the gap junctions (Acker et al. 2001; Irimia et al. 2002; Hunt et al. 2007) or from surface-catalysed nucleation at its periphery followed by cell-cell propagation (Toner et al. 1990; Acker et al. 1998), could lead to disruption of the cell cluster, affecting fate decisions, such as cell proliferation, differentiation and/or apoptosis on thawing. A number of recent studies reported increased levels of survival by protocols using seeding, in which ice is preferentially nucleated in the extracellular medium. This may result in the reduction in the extent of intercellular ice formation and propagation in the clusters (Ware et al. 2005; Yang et al. 2006), corroborating the hypothesis that IIF contributes to cryoinduced damage to hESC colonies.

Herein, entrapment within UHV alginate prior to freezing was used as a strategy to improve post-thaw recovery as it is a clinical-grade, xeno-free matrix which has already proven effective for the cryopreservation of cell monolayers and cell aggregates (Malpique et al. 2009; Malpique et al. 2010). Moreover, micro-encapsulation of hESC within alginate has already been shown as an effective strategy for prolonged feeder-free maintenance without affecting hESC pluripotency (Siti-Ismael et al. 2008). Our results show that entrapment beneath a layer of UHV alginate does not provide protection to slow-rate frozen as either adherent hESC colonies or colony clumps in suspension: after 24 hours post-

thawing the majority of the cells were dead beneath the alginate layer or inside the micro-capsules. Entrapment within extracellular matrix (ECM) or addition of ECM components have been shown to help adherent hESC colonies survive slow-rate freezing and thawing (Ji et al. 2004; Kim et al. 2004), which has been related to decrease post-thaw apoptosis (Ji et al. 2004; Heng et al. 2006). These studies suggest that it may be necessary to supplement the cryomedium with ECM components, such as collagen or laminin, in order to reduce delayed post-thaw cell apoptosis (Martin-Ibanez et al. 2008).

Aiming at reducing the cell death observed post-thawing in adherent hESC colonies, serum-free CryoStorTM solution was tested in this study as it has previously been shown to improve post-thaw recovery in a series of cellular systems (Baust et al. 2001; Baust et al. 2002; Sosef et al. 2005; Baust et al. 2006; Stylianou et al. 2006; Malpique et al. 2009; Malpique et al. 2010). CryoStorTM solution has been carefully formulated to maintain the ionic and hydraulic balances of cells at low temperatures and is supplemented with additional components to inhibit post-thaw apoptosis. We have also tested the addition of the Rho-kinase inhibitor ROCK and apoptosis inhibitor Z-VAD-FMK to the cryo- and post-thaw medium since these molecules have been suggested to reduce post-thaw apoptosis in hESC colonies (Heng et al. 2007; Martin-Ibanez et al. 2008). We have found no significant improvement occurred when CryoStorTM solution was used when compared with serum-free CM or CM containing 50% serum when hESC were cryopreserved as adherent colonies, either with or without alginate entrapment. The addition of ROCK and Z-VAD-FMK to the cryo- and post-thaw media was not able to increase post-thaw viability of hESC. These results suggest that post-thaw apoptosis mechanisms triggered by other pathways rather than caspase- and Rho-kinase intermediates may be involved in the loss of viability in hESC colonies.

Vitrification minimizes cell exposure to non-physiological temperatures and circumvents problems associated ice formation and cell dehydration, thus eliminating the risk of colonies fragmentation and the need for control of freezing rates and seeding temperature

In this work, we have investigated vitrification of adherent colonies as a novel strategy to eliminate the cell-clump selection and technical problems associated with vitrification of hESC clumps. Our results show that intact hESC colonies can be effectively cryopreserved in serum-free media through vitrification, yielding significantly higher hESC recovery rates when compared with the slow-rate freezing approach. Moreover, confluent hESC colonies were recovered with a high survival and low differentiation rates, comparable with those reported for vitrification of hESC clumps in close or open pulled straws (Reubinoff et al. 2001; Richards et al. 2004) or cell strainers (Li et al. 2008). Our surface-based vitrification method proved to be the best option for maintaining high cell viability and pluripotency of undifferentiated hESC after thawing.

By using the surface-based vitrification strategy described in this study we were able to circumvent the major technical drawbacks associated with vitrification of hESC clumps. Vitrification in thin-walled OPS (Reubinoff et al. 2001), electron microscopy grids, and small nylon loops (Mavrides et al. 2002), directly into liquid nitrogen, has been shown to be an effective approach for the cryopreservation very small number of hESC clumps (Reubinoff et al. 2001; Richards et al. 2004). However, these tiny carriers are not suitable for process scale-up since colonies must be very small (100 to 200 cells) and only a few colonies can be stored per straw due to heat transfer limitations. In 2008, Li *et al.* reported that bulk vitrification of hESC clumps in cell strainers is as effective as the open-pulled straw method (Li et al. 2008). However, this protocol involves loss of biomass as clumps with a diameter bellow 70 μm are excluded and it is still dependent on many factors including the cellular stress that occurs during colonies division into clumps and centrifugation of the cell pellet.

Colonies on culture plates possess a relatively low surface area to volume ratio, which makes it difficult to achieve the rapid cooling and warming rates required for vitrification protocols (Fahy et al. 1984). As a solution to overcome this, we add only a minimal volume of cryopreservation solution (Ure et al. 1992) to form a thin film of liquid to cover the adherent cell layer at the bottom of the wells, before submerging the base of the culture plate in liquid nitrogen. This allowed the high surface area to volume ratio necessary to achieve the rapid cooling rates that are required for vitrification. At the same time, the thin film of vitrification solution facilitated rapid thawing of the culture plate.

Still some limitations remain to be solved, namely the physical integrity of cryopreserved culture plates in long-term storage, which has not been determined at the temperature of the vapour-phase of LN₂, and the difficulty of maintaining sterile conditions in liquid nitrogen storage and during the thaw phase in a water bath. Filtration and ultraviolet radiation of LN₂ at cooling may protect against contamination (Lee et al. 2008). Another way to overcome this limitation and standardize the cryopreservation of hESC colonies is to redesign the cell culture plate. In the model proposed by Heng et al., a culture plate made of synthetic materials resistant to storage at -196°C is designed with readily attachable screw-cap culture wells (Heng et al. 2005). The detachable wells facilitate storage and after thawing can easily be reattached to a specifically designed holding plate.

Because intact hESC colonies are firmly attached to the surface of culture plates there is no need to use either manual selection or centrifugation of hESC clumps prior to freezing and post-thawing.

5. CONCLUSION

The present study shows that confluent, adherent hESC colonies in culture plates can be effectively cryopreserved in xeno-free conditions through vitrification. The vitrification method proposed herein is simple, efficient, quick and inexpensive since it does not involve cell manipulation prior to freezing or post-thawing and the use of a controlled-rate freezer is not required. The step-by-step transfer of the culture dish containing hESC colonies between freezing/thawing medium and out into LN₂ is simpler and faster comparing to cell clumps transfer by Pasteur pipettes thus decreasing transfer time and cell exposure to highly toxic CPAs. This method can cryopreserve a large amount of hESC at once without enzymatic or mechanical dissociation, which makes it ideally suited for use in assist the development of machine-automated systems for large-scale cryopreservation of bulk quantities of cells, reduce batch-to-batch variations and reduce time between cell storage and use in experimental or clinical settings. Furthermore, once the problem associated with the direct contact of the sample with LN₂ is solved, it will be suited for GMP – facilities and hESC banks.

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DISCUSSION AND CONCLUSIONS

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1. DISCUSSION

The success and availability of cell-based products for a range of applications, from pre-clinical research to biotechnology and reparative/regenerative medicine, depends, to a large extent, on the ability to successfully store samples until the time of use. Developing cryopreservation protocols and solutions to satisfy the demands of the different cell-based markets requires a multi-faceted approach which should be focused on both the sample specific requirements and its final application.

The work developed in this thesis aimed at overcoming critical challenges associated with the cryopreservation of complex cell-systems for cell-therapies and pre-clinical research. Distinct case studies were investigated and novel cryopreservation methodologies were developed and further optimized. For this purpose, several analytical tools were established to monitor important cell physiological and biological characteristics and also to assess their quantity and quality after cryopreservation. Gained knowledge on the effect of cryopreservation to each particular system studied revealed that each case faces unique challenges and evaluating them prior to cryopreservation is crucial to decide on the appropriate method to be used.

1.1. INTEGRATING CULTURE AND CRYOPRESERVATION METHODOLOGIES TOWARDS A FINAL-APPLICATION-FOCUSED PROCESS

1.1.1. Evaluating Process Requirements

When developing a cryopreservation protocol, the outcome, i.e. what the application calls for, should first be defined considering the sample characteristics, the needs of the application, and the method requirements in order to select the best option. Figure I illustrates the complexity of the relationships that one can established between challenges, strategies and final outcome.

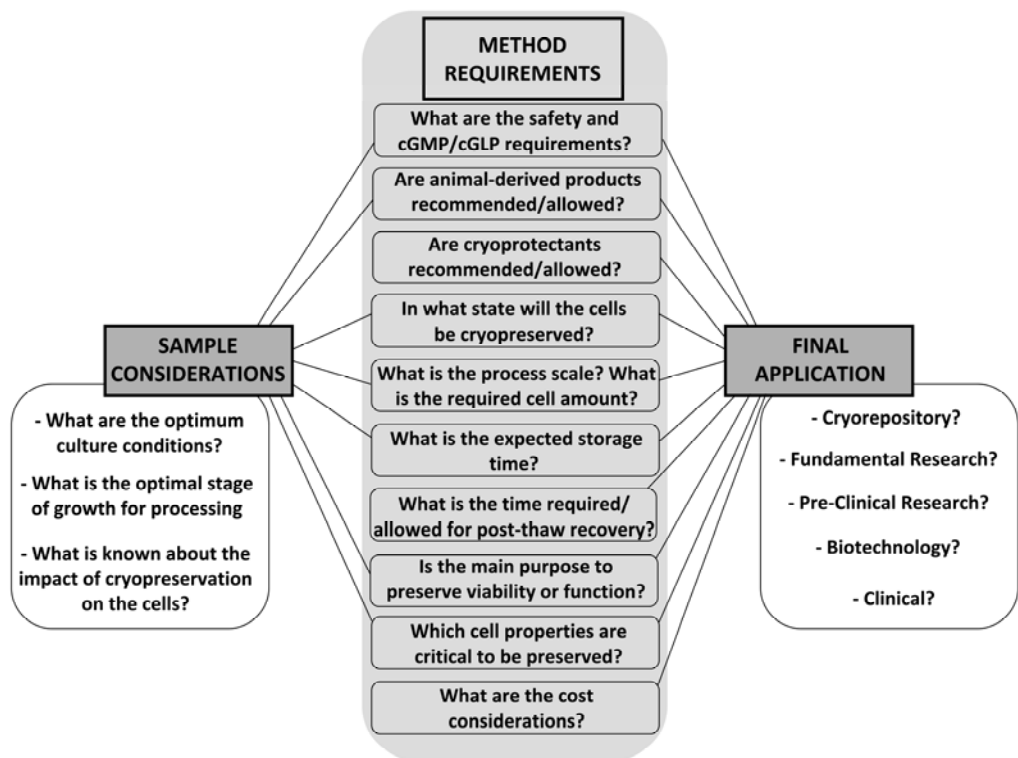


Figure I. Methodology Selection.

In this thesis, cryopreservation strategies were investigated for three cell types chosen for their unique features and potential applications: transformed cell lines with the capacity for spontaneous/induced differentiation (Chapters II and III); primary cultures of brain cells, which aggregate in suspension and differentiate into the major cell types found in the brain (Chapter IV); and pluripotent hESC (Chapter V). These cell types share an important culture requirement, which is of major importance for the final application and influences the choice of the cryopreservation method: the need for maintenance of cell-cell and cell-matrix connexions during culture in order for cell-specific function to be maintained. This requirement settled the state in which they ought to be cryopreserved, aiming at an integrated culture-cryopreservation process which reduces post-thaw manipulation until final application and facilitates process scale-up and automation:

- evaluation of process miniaturisation and automation through cryopreservation of functional Caco-2 single-cells in suspension (Chapter II);
- evaluation of an integrated process for the culture and cryopreservation of functional monolayers of Caco-2 and N2a cells for applications in pre-clinical research (Chapter III);
- evaluation of an integrated strategy allowing for long-term culture (in spinner vessels or bioreactors) and cryopreservation of primary brain cultures of neurospheres as 3-D aggregates in suspension for applications in pre-clinical research (Chapter IV);
- evaluation of the protocols developed in Chapters III and IV for the cryopreservation of pluripotent hESC as adherent colonies or colony clumps for pre-clinical research and therapeutic applications (Chapter V).

The aim for a cGLP/cGMP compliant process was also taken into account, namely the search for xeno-free reagents and avoidance of methods that might transmit infectious agents or add high batch variability into the process. When cells are intended for therapeutic applications, cGMPs compliance is required at steps from equipment and reagent selection to validation and use. Otherwise, cGLP compliance may be sufficient; some concerns should be addressed still, e.g. the avoidance of reagents/methods that may compromise process reproducibility.

1.1.2. Product Characterization - Establishing Methods To Assess Post-Thaw Cell Recovery

Due to the lack of accurate, validated methods for post-thaw assessment that matches the cell-systems used in this thesis, analytical tools were established for evaluation of product quality and process yield. Meaningful time points for assessment of post-thaw recovery were also evaluated since cells with intact PM, which are assumed to be viable immediately after thawing, may

not be able to fully recover their function and efficiently resume growth and metabolic activity (Frim et al. 1978; Baust et al. 1998; Baust et al. 2002).

As cells respond to cryoinduced damage by different mechanisms (e.g. osmotic shrinkage, mechanical damage to PM or protein denaturation) (Mazur 1970; Mazur 1984), recovery could not be defined in terms of a single physiological and/or morphological parameter. Thus, as a starting point, evaluation of recovery was performed through qualitative assessment of membrane integrity and apoptosis, and by the quantitative measure of metabolic activity using the alamarBlue assays which, contrary to other probes (e.g. MTT), allows the assessment of the same cell culture repeatedly over a nearly unlimited time period. Further analysis were carried out on the proliferation capacity and differentiation potential/state during long-term culture after thawing.

Electron microscopy constituted a crucial method for examining cellular features typical of a Caco-2 differentiated state (Pinto 1983) as demonstrated in Chapters II, III and IV. Cryoinduced damage to monolayers, such as shrinkage of “tissue-like” structures” or the presence of apoptotic cells, and maintenance of cell-cell connections (Borderie et al. 1998; Katsen et al. 1998; Komuro et al. 1999; Heng et al. 2006) could also be evaluated using this technique.

Metabolic activity status of the cultures, exchange of nutritional factors and patterns of morphological differentiation (Seeds et al. 1971; Honegger et al. 1977) were shown to be good indicators of the post-thaw culture state (Chapter IV). For a better assessment of neurosphere functionality, studies on spontaneous electrical activity (Stafstrom et al. 1980), and synthesis, storage and release of neurotransmitters (Honegger et al. 1979; Santos et al. 2007) should also be performed. However, due to time limitations these techniques were not pursued further.

Since post-thaw increase of hESC uncontrolled differentiation and loss of pluripotency have been reported (Reubinoff et al. 2001; Kim et al. 2004; Richards et al. 2004; Heng et al. 2006), methods to evaluate the effect of the cryopreservation process on hESC pluripotency after a freeze/thaw cycle and further passage were established (Chapter V). In addition, analysis of cell karyotype should also be performed to confirm maintenance of normal human chromosome status since post-thaw culture may artificially select for cells that are more robust withstanding the cryopreservation process - an often overlooked fact that can contribute to genetic drift (Baust et al. 2006). These assays are currently being done in collaboration with IPO (Lisbon) as they require exquisite methodologies.

In summary, a combination of assays was established to analyse cell recovery. These were designed to address multiple aspects of cryoinduced cell death and allowed the assessment of product quality and process yield. Moreover, the importance of evaluating proliferation, maintenance of cell function and differentiation potential during long-time culture after thawing was confirmed, which is a fundamental requirement for further cell-based applications.

1.1.3. Novel Cryopreservation Strategies for Complex Cell-Systems

Cryopreservation of Caco-2 and N2a monolayers and primary cultures of brain neurospheres through traditional methods, i.e. slow-rate freezing in serum-supplemented medium containing 10-20% DMSO (McLellan et al. 1995), caused severe cryoinduced damage, namely:

- loss of cell-cell and cell-matrix interactions resulting in cell detachment from the surface and cell loss from the aggregates. This was concomitant with (but not necessarily a consequence of) the loss of membrane integrity and metabolic activity, which started immediately after

thawing and continued over the following 24 hours up to 7 days depending on the cell type;

- occurrence of a post-thaw delay onset of cell-death, related to cryoinduced apoptosis as observed for Caco-2 and N2a cells.

These results, presented and discussed in Chapters III and IV, are in accordance with previous reports for different cell lines cryopreserved as monolayers (Hornung et al. 1996; Wusteman et al. 1997; Ebertz et al. 2004) and 3-D multicellular systems (Merchant et al. 1993; Purcell et al. 2003; Ehrhart et al. 2009) clearly confirming that of cryopreservation protocols for such systems cannot be simply adopted from those used for single-cell suspensions. Our results have also shown that both the maintenance of cell-specific interactions after thawing and the reduction of cryoinduced apoptosis are important requisites that need to be fulfilled in order to optimize the cryopreservation process for 2-D and 3-D cell-systems.

Strategies applied to overcome the bottlenecks found in the cryopreservation of each system and main achievements that resulted from this thesis are summarized in Table I.

Table I. Summary of the bottlenecks addressed, strategies developed and main achievements of the work developed in this thesis for the cryopreservation of complex cell-systems.

Aim	Bottlenecks	Strategies	Main Achievements
Functional Single-cell Suspensions for Pre-Clinical Research	Traditional storage volumes (1 ml cryovials); - Higher susceptibility to heterogeneous ice nucleation; - Incompatible with high-throughput cell banking; - Requires evaluation of the importance of assessing long-term post-thaw culture properties.	- Reduction of the cryovessel volume through the use of micro-cryosubstrates (25 µl/well); - Evaluation of automated system for CPA addition; - Evaluation of proper time points for evaluation of cell recovery using proper cell-specific indicators.	- Efficient cryopreservation in micro-cryosubstrates: no effect on post-thaw membrane integrity neither cell proliferation nor differentiation potential; - The importance of evaluating the effect of the cryopreservation process on cell function and differentiation potential was confirmed.
Integrated Culture-Cryopreservation of Functional 2-D Monolayers for Pre-Clinical Research	- Extensive membrane damage, cell detachment and up to 80% loss of metabolic activity within 24 hrs; - Extended lag phase until return to pre-freezing cell number and functional state.	- Entrapment beneath a layer of UHV alginate; - Cryomedium containing anti-apoptotic factors (CryoStor™).	<u>Combination of UHV alginate and CryoStor™:</u> - Strong inhibition of membrane damage and cell detachment; - Return to pre-freezing cell number and functional state achieved with reduced lag phase.
Integrated Culture-Cryopreservation of 3-D Aggregates for Pre-Clinical Research	- Membrane damage, aggregates fragmentation and up to 60% of metabolic activity within 24 hrs; - Complete loss of function and metabolic interactions within 7 days.	- Encapsulation within micro-capsules of UHV alginate; - Cryomedium containing anti-apoptotic factors (CryoStor™).	<u>Combination of UHV alginate and CryoStor™:</u> - 30% increase in metabolic activity and membrane integrity after 24 hrs; - Complete inhibition of disintegration and full recovery of metabolic activity within 7 days.
Integrated Culture-Cryopreservation of hESC Colonies for Pre-Clinical Research and Therapy	<u>Slow-rate freezing:</u> - Colony disintegration and/or detachment from the surface and loss of >90% viability; - Uncontrolled differentiation; - Loss of pluripotency; <u>Vitrification</u> - Technical limitations associated with vitrification of clumps in straws.	<u>Slow-rate freezing:</u> - Combination of entrapment of adherent colonies/clumps within UHV alginate and CryoStor™; - Addition of apoptosis inhibitors to the cryo- and post-thawing medium; <u>Surface-based vitrification:</u> - Vitrification of adherent colonies;	<u>Surface-based vitrification:</u> - Complete inhibition of colonies disintegration and/or cell detachment; - Maintenance of >80% viability within 24 hrs post-thawing; - Inhibition of uncontrolled differentiation; - Reduced lag phase until next passage; - Maintenance of pluripotency.

1.1.3.1. Entrapment within UHV Alginate

Approaches for the cryopreservation of cell suspensions or adherent cells in compatible matrices, such as extracellular matrix (ECM), ECM components or commercial hydrogels, have previously been reported for cell types which were shown to poorly survive such process, namely hepatocytes, pancreatic islets and hESC (Koebe et al. 1990; Zhou et al. 1997; Rialland et al. 2000; Mahler et al. 2003; Ji et al. 2004).

In this thesis, we show for the first time that entrapment within xeno-free, UHV, highly-purified alginate of clinical-grade is an effective strategy to avoid the observed loss of cell-cell/matrix interactions that results from cryopreserving 2-D monolayers of functional cell lines, primary cultures of 3-D aggregates through slow-rate freezing in culture medium with 10% DMSO.

Before assessing the effect of UHV alginate entrapment on post-thaw cell recovery, its biocompatibility with the three cell systems analysed was evaluated and confirmed. However, it should be pointed out the importance of alginate entrapment after a particular time of culture for each system so that proliferation and function are not affected: alginate addition over adherent cells should be performed after achievement of confluent Caco-2 monolayers, fully formed N2a neuronal networks or confluent hESC colonies; micro-encapsulation of neurospheres and hESC clumps should be performed after cell recovery from the shear force effects involved in tissue dissociation and adaptation to the culture system, which take place during the first days of culture (Hunt et al. 2007; Santos et al. 2007).

An improvement of 40% to 50% recovery (depending on the cell type) at 24 hours post-thawing was obtained when cells were entrapped within UHV alginate (Chapters III and IV). In Chapter III, alginate entrapment was shown to improve the recovery of undifferentiated Caco-2 and N2a monolayers cryopreserved in serum-supplemented culture medium by reducing membrane

damage and cell detachment and increasing metabolic activity immediately after thawing. In Chapter IV, micro-encapsulation was shown to avoid the post-thaw physical rupture and membrane damage of the neurospheres when compared with non-encapsulated neurospheres. Although being significant improvements for both cell-systems, the presence of 40% to 50% cells with damaged membranes beneath/within the alginate matrix observed within 24 hours post-thawing still limits the efficiency of the process due to an extended lag phase until the population replicates back to the same number originally cryopreserved. The system may return to 100% yield, but at the expense of time and materials. Moreover, many cells currently being used for cell therapy applications are terminally differentiated, i.e., lack the capacity to proliferate (e.g. neurons and hepatocytes), thus the cell number prior freezing will not be achieved again.

The observed delayed onset of cell death might be related to sequential apoptotic and necrotic processes not evident immediately subsequent to thawing (Frim et al. 1978; Baust et al. 2000) and is highly critical to consider for therapeutic applications, where thawed cells may appear viable immediately upon implant but succumb a day or later in the patient, resulting in transplant failure.

As we aimed for cryopreservation methodologies which assure minimum recovery/expansion period between thawing and final application, we searched for solutions which could reduce the delayed onset of cell death.

1.1.3.2. Cryomedium

The use of the serum-free CryoStorTM solution resulted in a considerable improvement of the cryopreservation process for both Caco-2 and N2a cells cryopreserved in an undifferentiated state. The combination of cell entrapment in UHV alginate and CryoStorTM solution lead to optimized protocols for the cryopreservation of cell monolayers, enabling 60% to 100% recovery of metabolic

activity and maintenance of proliferation and differentiation state/capacity as summarized in Table I.

Caco-2 and N2a are valuable, well-established *in vitro* models and high-throughput screening assays in the pharmaceutical industry (Blais et al. 1987; Bani-Yaghoub et al. 2005; Shah et al. 2006). For some applications, cryopreservation of both cell lines in a well-characterised, fully differentiated state, rather than undifferentiated, might be more interesting for pre-clinical research to avoid the long-term culture time post-thawing required for cell differentiation.

Fully differentiated Caco-2 cells form multi-layers on well-plates, which brings further implications to the cryopreservation process rather than just the presence of cell-cell and cell-substrate interactions in the monolayers, such as diffusion restrictions of water and CPA through the multilayer structure. These may be responsible for the lower recovery rates observed, implying increased recovery times until return to pre-freezing cell numbers when compared to undifferentiated monolayers. Thus, further studies should be performed to improve the cryopreservation of Caco-2 cells in a differentiated state so that they can be used as immediately after thawing as possible.

N2a cells were also cryopreserved as differentiated neuronal networks aiming at their direct application in neurophysiology, drug discovery or toxicity testing (Wiche et al. 1983; Mao et al. 2000). Although the use of CryoStorTM was shown to be enough to allow high recovery of viability for undifferentiated monolayers, UHV alginate entrapment was crucial for the maintenance of cell attachment and cell-cell interactions after 24 hours following thawing. The 60% viability obtained after freezing with our optimized conditions is comparable with previous results in literature for adherent neuronal networks (Ma et al. 2006). We further assessed cell recovery within the following hours to days post-thawing, which is of major importance for the evaluation of the full extent of post-thaw cell death due to the time course of apoptotic and necrotic mechanisms (Frim et al.

1978; Baust et al. 2000; Baust et al. 2002). Electrophysiological studies should be conducted to further confirm the functionality of cryopreserved neuronal networks.

Overall, the combination of different strategies based on cell entrapment in UHV alginate and serum-free CryoStorTM solution lead to optimized protocols for the cryopreservation of cell monolayers, which enabled the retention of cell membrane and whole monolayer integrity, metabolic activity and cell specific function/differentiated state. Successful cryopreservation of large numbers of viable and functional adherent cells on micro-plates eliminates the time-consuming process of inoculation and expansion from a frozen vial of cells, thus reducing time between cell storage and use in experimental settings. Furthermore, such protocols as developed here facilitate the creation of cellular clone banks, reducing batch-to-batch variability and normalizing passage age, which are obstacles for achieving reliable results in pharmacological testing (Sambuy et al. 2005). These protocols also support the implementation of routine cryopreservation practices during preparation of engineered cells and tissues for clinical applications towards the immediate availability of cells grown on biocompatible matrices for transplantation to treat several injuries (e.g. cartilage, skin, cornea, etc) (Brunette et al. 2001; Marijnissen et al. 2002; Ebertz et al. 2004).

In Chapter IV, the combination of alginate micro-encapsulation and the use of CryoStorTM as the vehicle for the CPA allowed for the development of an integrated strategy for the successful long-term culture of neurospheres in stirring conditions and its cryopreservation with approximately 80% recovery immediately after thawing, 30% increase in viability within 24 hours post-thawing and increased recovery in metabolic activity and neuronal-astrocytic enriched culture behaviour within 3 days of culture post-thawing. Although this is a considerable improvement when compared to the conventional protocol (Purcell et al. 2003), a 3-days recovery time is still required before neurospheres have returned to culture behaviour comparable to non-frozen cultures. Moreover, metabolic

activity found for alginate-entrapped cryopreserved cultures was always below the activity found in non-frozen controls, which leaves room for improvement of cryopreservation conditions. Nevertheless, these are very encouraging results as they provide a basis for further investigation using alginate and brain aggregates in bioreactors, allowing long-term experiments under precisely controlled conditions to investigate brain cell metabolism (Santos et al. 2007).

The culture of large amounts of neurospheres which can be cryopreserved for creation of cellular clone banks eliminates the laborious, time-consuming process of cell isolation and expansion from the animal and further cell culture and complete neurosphere differentiation. It also decreases batch-to-batch variation, which is an obstacle for achieving reliable results in neurotoxicology testing. Moreover, efficient long-time culturing within an alginate matrix supports routine cryopreservation practices towards the implementation of tissue banks of neuronal stem cell spheroids for tissue engineering and clinical applications of spheroid-based CNS support system.

1.1.3.3. Combined Strategies for the Cryopreservation of hESC

The developed cryopreservation strategies for 2-D and 3-D cell-systems could not be successfully applied for hESCs. However, high recovery yields of undifferentiated colonies could be achieved when surface-based vitrification was applied over adherent colonies in culture plates instead of slow-rate freezing.

The suitability of either conventional slow-rate freezing or vitrification for cryopreservation of hESC has been the focus of an emerging debate (Hunt et al. 2007).

We have chosen slow-rate freezing as the primary freezing method in our studies as it is the conventional method routinely used in research and clinical centers and industry for most cell types and compatible with commercial freezing systems. However, slow-rate freezing yields very low cell survival for hESC and comparative studies have reported on a significant increase in recovery rates

through vitrification of hESC clumps, which have made this the method adopted by the majority of groups deriving hESC lines, despite the many practical difficulties associated (Reubinoff et al. 2001; Kim et al. 2004; Richards et al. 2004; Zhou et al. 2004; Hunt et al. 2007).

Thus, in Chapter V, combined strategies for the cryopreservation of hESC through the standard slow-rate freezing protocol were compared with a novel vitrification strategy for adherent hESC colonies. It was found that entrapment beneath a layer of UHV alginate that resulted so well for the cell-systems investigated in Chapters III and IV, does not provide further protection to hESC cryopreserved through slow-rate freezing as either adherent colonies or clumps, whether culture medium or CryoStorTM solution are used as the cryopreservation medium. Furthermore, although the apoptosis inhibitors tested are able to improve maintenance of cell attachment to the culture surface, loss of membrane integrity within 24 hours post-thawing in the majority of the frozen/thawed colonies is still observed.

On the other hand, vitrification of adherent hESC colonies on the top of ihFF feeders in gelatine-coated culture dishes yielded significantly higher recovery rates when compared to the slow-rate freezing approach. Such protocol resulted in low differentiation rates and maintenance of pluripotency post-thawing as denoted through hESC positive staining with pluripotent markers. Moreover, it overcomes the technical limitations associated with vitrification of hESC in straws, which makes them unsuitable for process scale-up since colonies must be very small (100 to 200 cells) and only a few colonies can be stored per straw (Hunt et al. 2007).

This novel surface-based vitrification method may reduce the time required to amplify frozen stocks and facilitate the development of a high-throughput cryopreservation process for hESC, thus supporting the widespread use of these cells in pre-clinical research, with minimum expense of time and

materials as cells may be assayed immediately after thawing on the same plates on which they were propagated and frozen.

For clinical applications, the number of cells required falls in the range of tens of million to a few billion (Kehoe et al. 2009). The robust, scalable production of such quantities of cells using bioreactors and cell culture in microcarriers have been employed (Kehoe et al. 2009; Nie et al. 2009). Vitrification of adherent cells, as presented in this work, is compatible with preservation on microcarriers or in micro-scale gel particles, which might provide the advantages of freezing at higher densities after cell culture in bioreactors.

Some issues remain to be solved as discussed in section below.

1.1.3.4. Process Automation and Miniaturisation

Aiming at sample applications for high-throughput pre-clinical research assays, the possibility for automation and process scale-up must be taken into account. Namely, the feasibility of culturing and cryopreserving large numbers of monolayer-containing well-plates or cell/aggregates in suspension. In Chapter II, the use of micro-cryosubstrates was evaluated for its potential towards process automation and miniaturisation. Micro-cryosubstrates were shown to be as effective as the traditional 1 ml cryovials for the cryopreservation of Caco-2 cells at different CPA concentrations. This strategy facilitates the establishment of cGMP compliant-processes as it allows to physically attach a cryo-tolerant memory chip to each cryosubstrate so that each sample's data is stored at-sample on-chip (Ihmig et al. 2006), which is especially important in the establishment of large cryobanks for sample repository.

The use of micro-cryosubstrates also allow the fast and exact addition of the CPA by using a pipette robot system, which results in an immediate and homogeneous distribution of the cryoprotectants and allows to easily deal with a large number of samples, has shown to be effective for Caco-2 cells cryopreservation. This pipette robot system may be used for addition of

cryomedium and CPA prior to freezing as well as CPA dilution in cryomedium after thawing of the micro-cryosubstrates. Moreover, it can be easily applied for automation of the cryopreservation process for cell culture well-plates.

Finally, the use micro-cryosubstrates brings also biophysical advantages as it avoids large heterogeneities (Zimmermann et al. 2003; Zimmermann et al. 2004; Zimmermann et al. 2005). When freezing takes place in large cryovessels and seeding is not induced in all samples at a pre-determined temperature, which is usually the case when commercial, simple freezing systems are used, large temperature gradients and heterogeneous ice formation occur, within the same cryovessel or for different samples in the same batch, as a result of spontaneous ice seeding at different points or different temperatures.

1.1.4. Understanding the Mechanisms of Cryoinjury / Cryoprotection in Complex Cell-Systems

Based on the work presented in this thesis, some speculation was given on the mechanisms of cryoprotection provided by the cryopreservation strategies purposed to each cell-system investigated.

Extensive cell detachment and membrane damage starting immediately after thawing have been reported for adherent cells, although it is not yet clear whether detaching or freezing caused injury (probably both factors play a role) (Hornung et al. 1996; Wusteman et al. 1997; Acker et al. 1999; Pegg 2002; Ebertz et al. 2004). Although the underlying mechanisms are far from being clearly understood, the contribution of different factors has been proposed, such as high prevalence of intracellular ice formation (IIF) mediated by adhesion contacts which leads to plasma membrane (PM) disruption (Berger et al. 1996; Yang et al. 1996; Acker et al. 1998; Acker et al. 1999; Acker et al. 2001). Moreover, the extended morphology of attached cells may create conditions for tension from local adhesions of the cell to the substrate during osmotic shrinkage, rendering

the membrane more fragile to rupture. Cell adhesion may also result in damage to the ultra-structure (cytoskeleton, focal adhesions or gap junctions) due to mechanical forces, such as stretch, strain, and tension resulting from the differential thermal contraction between cells and substrates (Liu et al. 2005; Liu et al. 2006), which have been shown to render cells more susceptible to apoptosis (Hsieh et al. 2005).

Cryopreservation of cell aggregates presents additional concerns related with heat and mass diffusion restrictions within the aggregate (Karlsson et al. 1996) and ice crystals formation within the tissue, mechanically deforming cells and disrupting the intercellular interactions that are needed to maintain the functional 3-D tissue architecture of spheroids (Karlsson et al. 1996).

Our results suggest that alginate entrapment to both 2-D and 3-D systems improves cell recovery by providing cell immobilisation thus avoiding detachment from the surface/aggregate and breakage of cell-cell interactions. Furthermore, as a considerable increase in the number of attached cells with undamaged membranes was found immediately after thawing, it seems plausible that the presence of the gel not only avoids physical cell rupture but also mitigates cell damage. The fact that 30% to 40% cell death still occurs within 24 hours post-thawing for both cell-systems, which is comparable to non-encapsulated cultures, indicates that alginate protection is obtained by overcoming freeze-induced cell death primarily due to IIF and/or chemo-osmotic stress, which results in PM disruption and subsequent necrosis (Paynter et al. 1997), rather than inhibiting cryoinduced initiation of apoptotic pathways.

Although the mechanisms underlying the hydrogel's cryoprotective action have not yet been clearly elucidated, such effect may be related with the influence of the polymeric matrix of the gel on extracellular ice formation (Clegg et al. 1982; Murase et al. 2004) which causes mechanic constraints to the cells and influences IIF (Mazur et al. 1972; Toner et al. 1993), or buffering for the cryoprotectant's diffusion (Tan et al. 1998).

Alginate concentration and incubation time in cross-linking ion was shown to influence the cryopreservation outcome: 0.7% alginate was shown to be effective for the cryopreservation of cell monolayers, whereas decreasing alginate concentration to 0.4% was required to increase the post-thaw viability of micro-encapsulated cells in suspension. Both concentrations were shown to be adequate for the transfer of differentiation factors, nutrients and products of the metabolism. To understand the effect of the alginate concentration on the post-thaw viability of both cell types, further studies should be conducted, namely evaluation of alginate concentration on final viscosity and water / CPA diffusivity within the matrix. Heat and mass diffusion limitations and ice front migration within the alginate matrix are also known to impact the cryopreservation outcome of encapsulated cells (Karlsson et al. 1996; Cui et al. 2002). In the future, more fundamental studies on the physicochemical and biophysical phenomena occurring during freezing/thawing of alginate entrapment will allow for a further improvement of this process.

CryoStorTM protection is more likely to be involved in inhibition of necrotic and apoptotic cell death pathways. CryoStorTM has been carefully formulated to maintain the ionic and hydraulic balances of cells at low temperatures thus facilitating the preservation of cell homeostasis and control of the ionic environment and is supplemented with several additional components to reduce the generation of free radicals and inhibit apoptosis (Taylor 1982; Taylor et al. 1985; Taylor et al. 2001; Baust et al. 2002).

The use of CryoStorTM improves post-thaw recovery for both monolayers and neurospheres as compared to standard culture medium: high post-thaw membrane integrity and metabolic activity were maintained immediately after thawing and during further post-thaw culture, either with or without alginate gel entrapment. One can hypothesise that a decrease in post-thaw necrotic and/or apoptotic cell death pathways is related with the improved post-thaw recovery found for the two cell lines used in the present study. The fact that for both

differentiated N2a neuronal networks and neurospheres the use of CryoStor™ alone was not sufficient to avoid cell loss from the surface/aggregate and loss in viability within 24 hours post-thaw suggests that other mechanisms are involved that cannot be addressed by CryoStor™ alone and requires the presence of alginate to reduce detachment and improve viability.

It has been speculated that the basis for the disparity between the post-thaw recovery of mESC and hESC following slow-rate freezing lies in the “highly co-operative nature” of these cells. The presence of functional gap junctions in hESC, implicated in cell proliferation, differentiation, and apoptosis (De Maio et al. 2002; Sathananthan et al. 2002; Wong et al. 2004), has added weight to this theory (Hunt et al. 2007). Damage to the hESC clusters caused by intracellular ice propagation, either by random nucleation events within the cluster and propagation through the gap junctions (Acker et al. 2001; Irimia et al. 2002) or from surface-catalysed nucleation at its periphery followed by cell-cell propagation (Toner et al. 1990; Acker et al. 1998), could lead to disruption of the cell cluster, affecting both cell proliferation, differentiation and apoptosis on thawing. In such case, cryoinduced damage to hESC colonies would be related to IIF, which is corroborated by a number of recent studies indicating that increased levels of survival may be obtained by seeding (Ware et al. 2005; Yang et al. 2006). When seeding is performed, ice is preferentially nucleated in the extracellular medium which may result in the reduction in the extent of intercellular ice formation and propagation in the clusters (Ware et al. 2005; Yang et al. 2006), corroborating the hypothesis of intracellular ice formation contributes to cryoinduced damage to hESC colonies. Our results support this hypothesis since vitrification, which completely avoids IIF through induction of a “glassy-state”, significantly improved hESC recovery.

Whether apoptosis or necrosis is the primary mechanism of death in frozen/thawed hESC colonies is not yet clear. Our studies have shown that the use of CryoStor™ or molecules which were suggested to inhibit post-thaw apoptosis

following slow-rate freezing of hESC (Heng et al. 2007; Martin-Ibanez et al. 2008), were not able to increase post-thaw viability of the adherent colonies. These results suggest that post-thaw apoptosis mechanisms triggered by other pathways rather than caspase- and Rho-kinase intermediates may be involved in the loss of viability in hESC colonies.

1.2. IMPROVING CRYOPRESERVATION STRATEGIES FOR COMPLEX CELL SYSTEMS: WHAT CAN STILL BE DONE?

The work presented herein has shown that, by addressing the main bottlenecks in the cryopreservation of complex cell-systems, improved cryopreservation methodologies for such systems could be obtained. However, some issues remain to be solved and the developed strategies can be further addressed towards process optimization and full characterization as summarized in Table II.

Table II. Summary of the future perspectives for cryopreservation of complex cell-systems.

Aim	Issues to be Solved / Future Work
Integrated Culture-Cryopreservation of Functional 2-D Monolayers for Pre-Clinical Research	<ul style="list-style-type: none"> - Evaluation of micro-cryosubstrates for adherent cell (increase recovery and reproducibility through heterogeneous nucleation; process automation); - Understand cryoinjury mechanisms for confluent, fully differentiated Caco-2 multilayers and N2a neuronal networks to further improve post-thaw recovery.
Integrated Culture-Cryopreservation of 3-D Aggregates for Pre-Clinical Research	<ul style="list-style-type: none"> -Understand the effects of cryoprotection through alginate entrapment / CryoStor™ to reduce lag phase during post-thaw culture; - Cryopreservation in micro-cryosubstrates (increase recovery and reproducibility through heterogeneous nucleation; process automation).
Integrated Culture-Cryopreservation of hESC Colonies for Pre-Clinical Research and Therapy	<ul style="list-style-type: none"> - Redesign of culture plates to avoid direct contact with LN₂, increase storage capacity and allow process scale-up and automation. - Understand cryoinjury mechanisms for hESC through slow-rate freezing to establish optimized protocols for high-throughput cryopreservation of colony clumps.

For pre-clinical research, the biophysical advantage of the micro-cryosubstrates when compared with cryovials should be evaluated through their potential to reduce post-thaw cell death for primary cultures of neurospheres or hESC clumps is suspension, which are more prone to cryodamage. Moreover, micro-cryosubstrates with a modified surface (e.g. micro-patterned substrates for cell differentiation) should be evaluated for culture and cryopreservation of adherent cells. Cryopreservation in micro-cryosubstrates will allow for improved reproducibility and process automation as well as increased storage capacity. Although cryopreservation in micro-cryosubstrates is not compatible with freezing

bulk quantities of cells, it may be the best option for storage of a great variety of samples or for easy evaluation of the best freezing conditions.

For clinical applications, vitrification of well-plates containing hESC might require re-design of the culture plates as plunging the cells directly into LN₂ is not compatible with cGMP practices. Moreover, methods for vitrifying hESC colonies adherent to microcarriers should be evaluated aiming for an integrated process for hESC expanded in bioreactors.

Finally, new cryomedium formulations must be less toxic than current formulations using DMSO and the use of xeno-free products should be extended to culture conditions upon rewarding whenever that is required before cells application into the patient. Although the complete replacement of DMSO by the non-toxic agent glycerol has shown not to be advantageous for Caco-2 cells cryopreservation, the results presented herein indicate that it is possible to reduce the DMSO concentration from 10% to 5%, which should be further investigated for hESC to be used in clinical applications.

Improving cryopreservation methodologies must focus not only in overcoming technical challenges towards a more robust process, but also understanding the mechanisms involved in cryoinjury to the cell-types examined, as well as the mechanisms by which the the proposed strategies improved cryoprotection. Based on the work presented in this thesis, some speculation was given on the mechanisms of cryoprotection provided by the cryopreservation strategies purposed to each cell-system investigated. As activated cold-induced stress pathways have been demonstrated to be cell-specific, the future of improved cryopreservation protocols may demand a portfolio of solutions designed to match and manage those cell-specific stress pathways. One path is to use a proteomic and genomic approach and different cell models to understand the cell-specific stress pathways that are activated as a consequence of cryopreservation. Taking these results into consideration, tailor-based

cryopreservation methodologies can be developed to best fit the cells and tissues into their different cell-based markets.

2. CONCLUSIONS

This thesis presents novel and effective strategies for cryopreservation of complex cell-systems, some of each allowing for an integrated process in which cells may be processed from culture to cryopreservation and final application with minimum expense of time and means. The results presented herein show that there is no one “universal” cryopreservation method that fits all different cell types and applications. However, by evaluating the complexity of the cell-system and the specific challenges concerning both the impact of the cryopreservation process on cell recovery and the requirements of the final application, one can identify one or more suitable strategies for a robust, cost-effective cryopreservation process.

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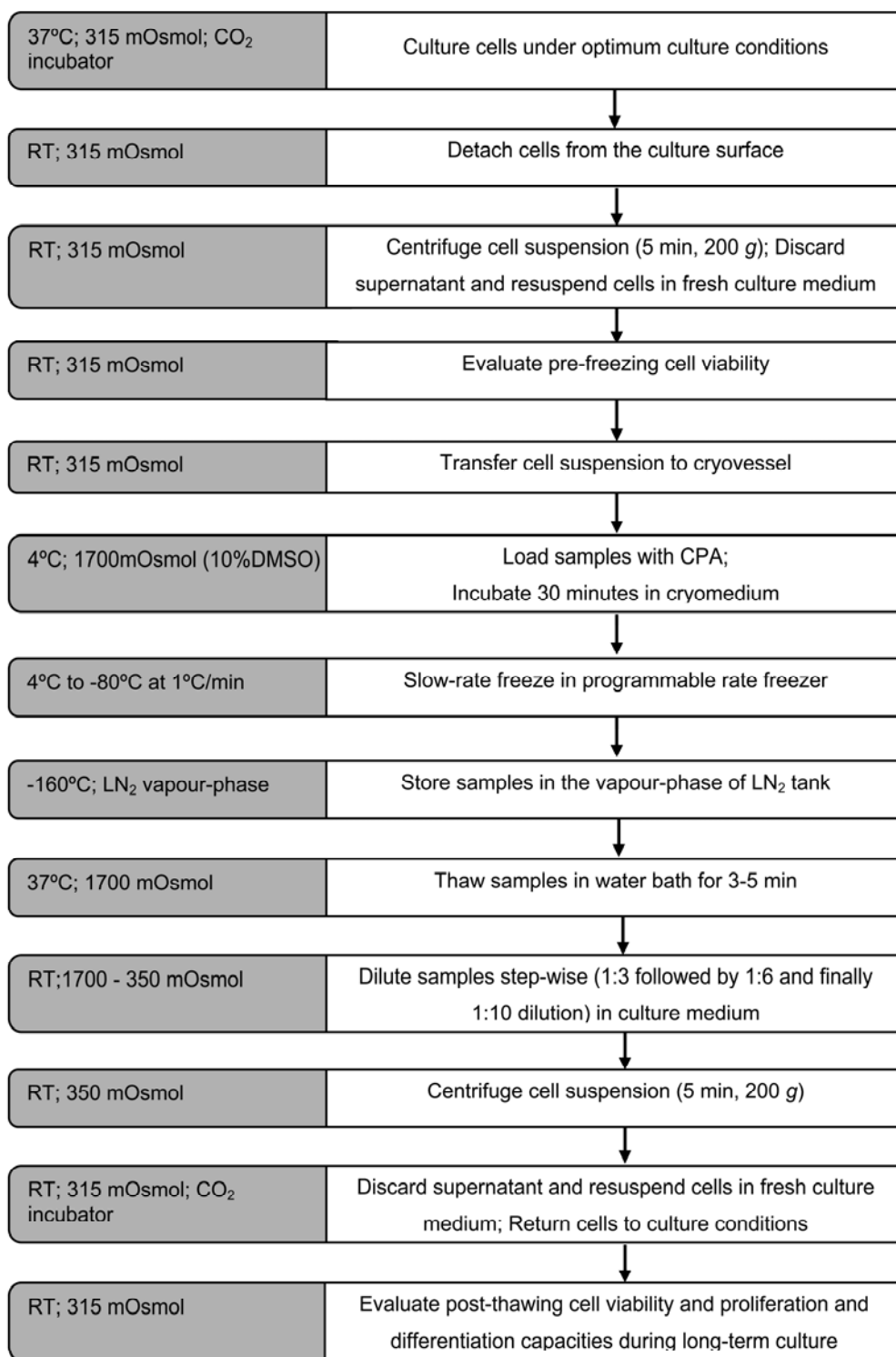
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APPENDIX.

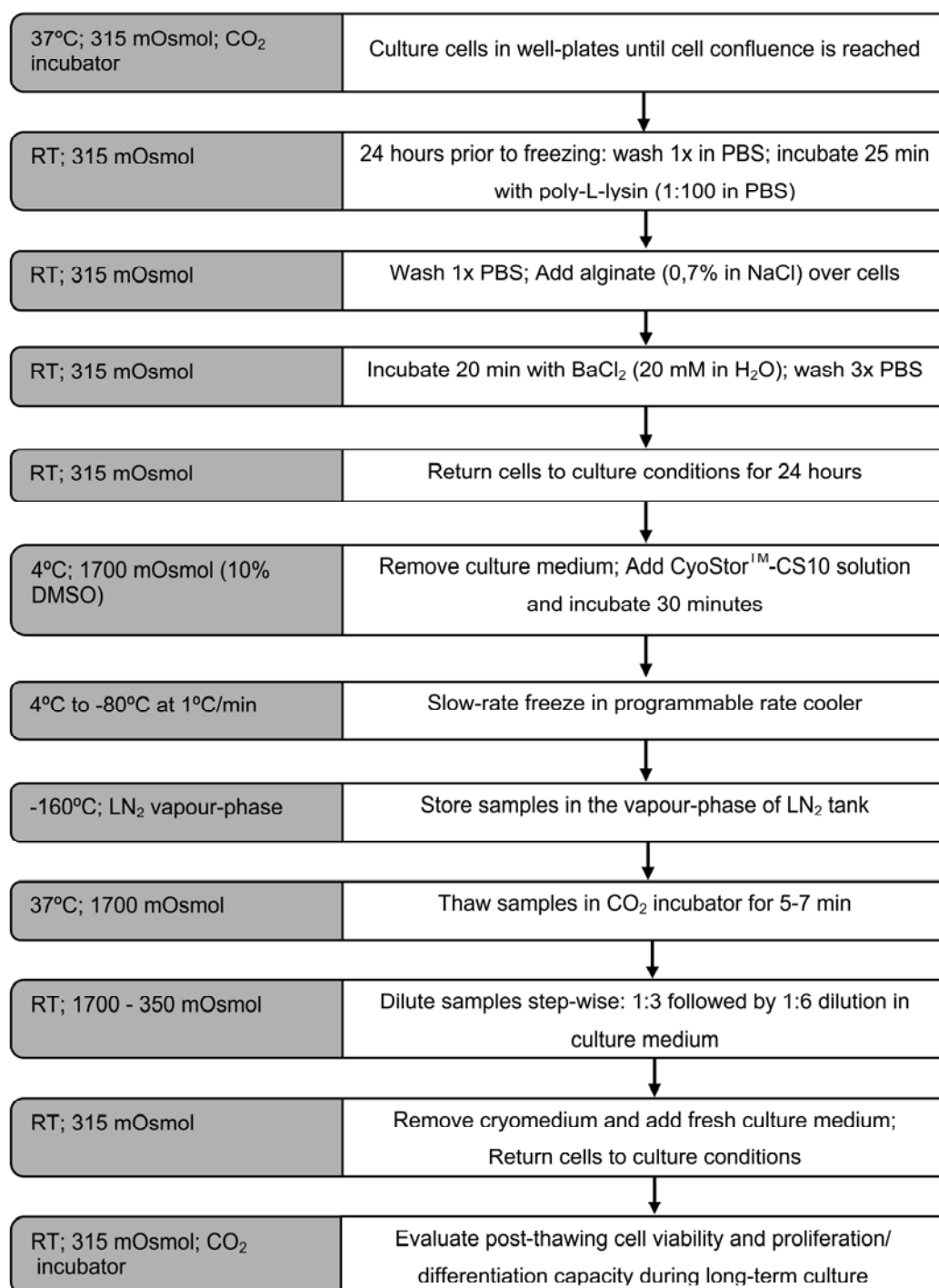
CRYOPRESERVATION PROTOCOLS

ESTABLISHED IN THIS THESIS

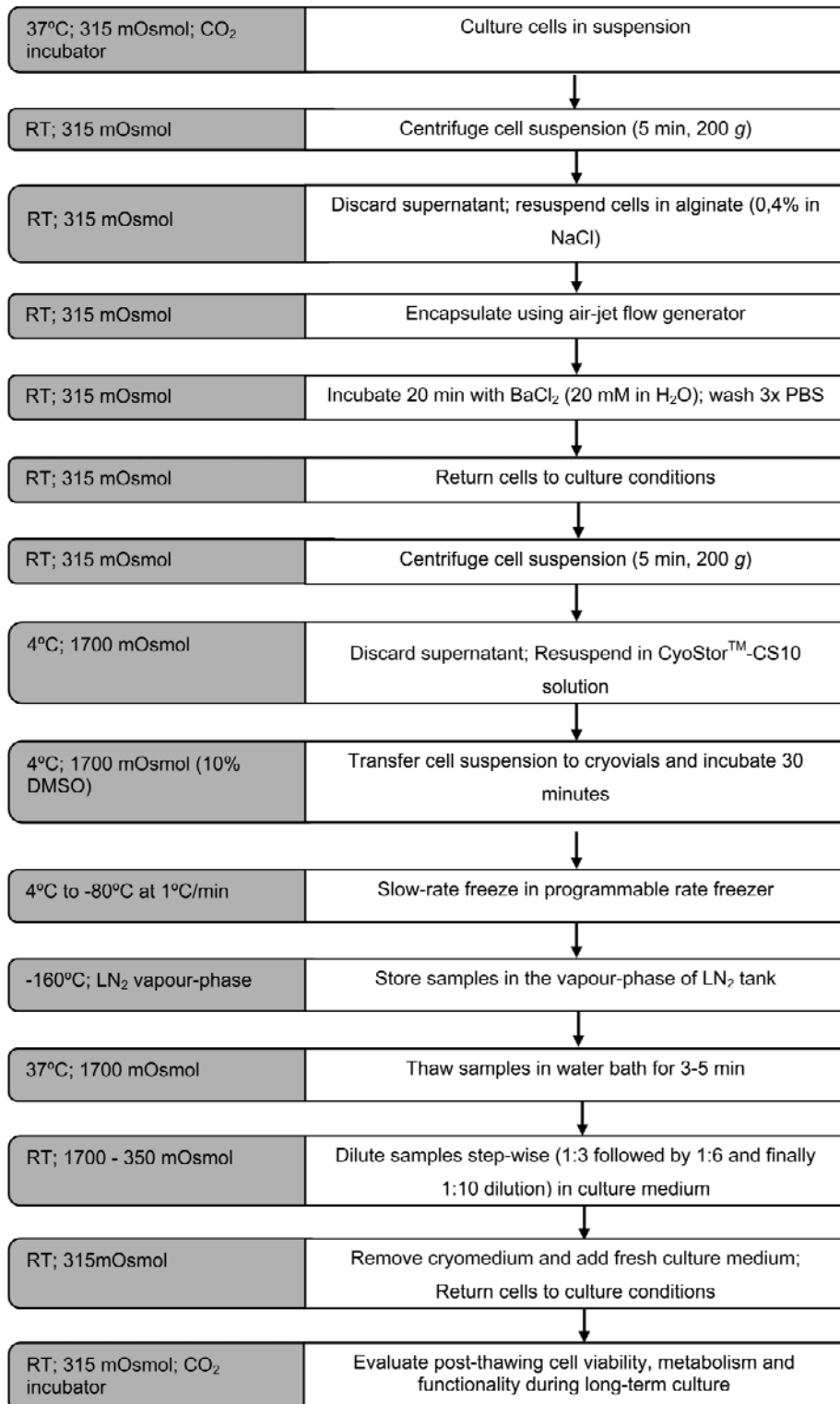
CRYOPRESERVATION PROTOCOL FOR SINGLE-CELLS IN SUSPENSIONS – SLOW-RATE FREEZING



CRYOPRESERVATION PROTOCOL FOR 2-D CELL MONOLAYERS – SLOW-RATE FREEZING



CRYOPRESERVATION PROTOCOL FOR 3-D CELL AGGREGATES – SLOW-RATE FREEZING



CRYOPRESERVATION PROTOCOL FOR hESC COLONIES - VITRIFICATION

